MOLECULAR DETECTION OF DENGUE VIRUS SEROTYPES FROM CLINICAL SAMPLES PREVALENT IN AND AROUND COIMBATORE

Dissertation Submitted in

Partial Fulfilment of the Regulations Required for the Award of

MASTER DEGREE in MICROBIOLOGY

BRANCH-IV

THE TAMIL NADU DR. M.G.R. MEDICAL UNIVERSITY

CHENNAI

MAY – 2018

PSG INSTITUTE OF MEDICAL SCIENCE & RESEARCH
PEELAMEDU, COIMBATORE – 641 004

CERTIFICATE

This is to certify that the dissertation titled **'MOLECULAR DETECTION OF DENGUE VIRUS SEROTYPES FROM CLINICAL SAMPLES PREVALENT IN AND AROUND COIMBATORE'** is an original work done by Dr.Thenmozhi Palanisamy, Post graduate student, during the period of her post-graduation in Microbiology in our institution. This work is done under the guidance of Dr.B.Appalaraju, Professor, Department of Microbiology, PSG Institute of Medical sciences and Research, Coimbatore.

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SELF DECLARATION

I hereby declare that this dissertation entitled “MOLECULAR DETECTION OF DENGUE VIRUS SEROTYPES FROM CLINICAL SAMPLES PREVALENT IN AND AROUND COIMBATORE” was prepared by me under the guidance and supervision of Dr.B.Appalaraju, Professor, Department of Microbiology, PSG IMS&R.

This dissertation is submitted to The Tamilnadu Dr. MGR Medical University in fulfilment of the university regulations for the award of MD Degree in MICROBIOLOGY.

Dr.Thenmozhi Palanisamy

ACKNOWLEDGEMENT

I would like to express my sincere gratitude to Dr.S.Ramalingam, Dean, PSG Institute of Medical Sciences and Research for allowing me to do my dissertation.

I wish to express my deep gratitude and appreciations to Professor and HOD of the Department Dr. B. Appalaraju., for his patient guidance, enthusiastic encouragement and useful critiques of this research work.

I am also extremely grateful to Professors and Assistant Professors for their valuable inputs and constant guidance while carrying out the study.

I express my sincere thanks to PSGIMS&R ethical and research Committee for their approval and financial assistance.

I would also like to thank all my colleagues, family members and department staffs in PSGIMS&R for their support and help that made this endeavour possible.

CONTENTS

INTRODUCTION

Dengue fever is a mosquito-borne tropical disease caused by the Dengue virus. Dengue fever virus (DENV) is an RNA virus of the family Flaviviridae; genus Flavivirus. Dengue is spread by several species of mosquito of the Aedes type, principally Ae. Aegypti and Ae.albopictus.

Aedes female mosquitoes are the principal vectors for the transmission of Dengue virus. They have many characteristics that make them suitable for transmission of the virus[1,2]. *Ae.Aegypti* usually breed in or close to houses, laying eggs in both man-made and natural water containers and their flight distance is relatively short. Their post-blood meal flight distance was up to a maximum of 441m as proposed by Reiter et al. in 1995. They are daytime feeders that prefer to bite humans and are frequently unnoticed. They can easily move on to another host, frequently taking multiple blood meals in a single breeding cycle[3]. Thus, an infected A. Aegypti mosquito may transmit Dengue virus to several individuals in a small area. For these reasons, family members, typically women and young children are at particularly high risk for infection. They are widely distributed in tropical and subtropical areas from latitude 45º North to 35º South.

Ae. albopictus mosquitoes, also called as Asian Tiger Mosquito. They are also a competent vector for the transmission of Dengue virus under both experimental and natural conditions. Their usual flight distance is about 200m. They are more tolerant to cold and have a wider geographic distribution than *Ae. Aegypti*[4,5]. However, they are less likely to transmit since they do not bite humans as frequently as *A. Aegypti* and appear to be less efficient natural vectors for Dengue virus. Large outbreaks are rare in regions that

have *Ae. albopictus*. Both *Ae. albopictus* and *A. Aegypti* are also competent vectors for transmission of chikungunya virus and led to simultaneous outbreaks of both diseases in some areas[6].Other Aedes mosquitoes appear to play an insignificant role in the global transmission of Dengue virus[7].

There are five closely related but serologically distinct Dengue viruses, called DENV-1, DENV-2, DENV-3, DENV-4 and DENV-5 of the genus Flavivirus. There is transient and weak cross-protection among the serotypes; therefore, individuals living in an area of endemic Dengue can be infected with different Dengue serotypes in a lifetime. Multiple virus serotypes often co-circulate within the same region (hyper endemicity), causing periodic epidemics.

The symptoms of Dengue infection usually begin three to fourteen days after infection and they include a high fever, headache, vomiting, muscle, joint pains, and a characteristic skin rash. Recovery generally takes two to seven days. In few cases it develops into the life-threatening Dengue haemorrhagic fever leading to bleeding, low levels of blood platelets and blood plasma leakage, or into Dengue shock syndrome.[8,9]

The diagnosis of Dengue fever may be confirmed by microbiological laboratory testing. This can be done by virus isolation in cell cultures, nucleic acid detection by PCR, viral antigen detection (such as for NS1) or specific antibodies (IgM and IgG serology).

AIM AND OBJECTIVES

AIM:

- To identify the Dengue serotypes using molecular methods and its association with disease severity.

OBJECTIVES:

i) To find out the proportion of Dengue infection in fever patients in hospital

ii) To classify primary and secondary Dengue.

iii) To find out the serotypes prevalent here using multiplex-nested PCR AND severity of the disease with serotypes.

REVIEW OF LITERATURE

HISTORY:

The first case of Dengue fever was from the Jin Dynasty (265-420AD), referred to a ‘water poison’ associated with the flying insects. The word Dengue may be derived from the Swahili phrase "Ka-dinga pepo", meaning "cramp-like seizure caused by an evil spirit". The word "Dinga" may have its origin from the Spanish word "Dengue" which means fastidious or careful, describing the gait of a person suffering the bone pain of Dengue fever[10].

Due to increased globalization secondary to slave trade there was an increase in the spread of Dengue in Africa during the 15th to 19th centuries. The first confirmed case report was in 1789 by Benjamin Rush, who coined the term "break bone fever" because of the symptoms of myalgia and arthralgia[11].

The primary vector, Aedes Aegypti leads to various epidemics from time to time. Transmission of Aedes mosquitoes was confirmed in 1906. Dengue was the second disease caused by a virus (after yellow fever). Ecological disruption leads to an increase in the marked spread of Dengue during the Second World War. The same trends led to the spread of different serotypes of Dengue virus to different areas and also to the emergence of Dengue haemorrhagic fever. This severe form was first reported in Philippines in 1953. In 1970s Dengue became the major cause of child mortality. First noted case of Dengue haemorrhagic fever and Dengue shock syndrome in central and South America was in 1981[12].

The possible factors that lead to the spread of Dengue fever include,

Overpopulation in unplanned urban areas leads to improper housing, water, sewage and waste management. Water stagnation leads to mosquito breeding and poor vector control. Changes in weather and evolution of virus leads to poor control of the disease. Frequent travel to endemic areas leads to spread of infection.

DENGUE VIRAL STRUCTURE:

The Dengue virion is a spherical particle existing as either a 50nm diameter immature 3 particle or a mature 60nm diameter particle. It has a lipopolysaccharide envelope. Dengue genome (11kb) has a single open reading frame and encodes three structural and seven non-structural proteins. The structural proteins are capsid(C), membrane (M), and envelope (E). The non-structural proteins include NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 (Halstead 2008). The Dengue particle consists of the genomic RNA surrounded by the capsid, then the envelope with E and M proteins bound (zone 2012). Mature and immature viruses can be distinguished based on these proteins. The mature virus is referred to as ‘spiky’ as M proteins bound to a precursor membrane protein (pr) form heterodimers with E proteins that appear as ‘spikes’ on the viral surface. In mature virions the soluble pr is cleaved from M protein by furin, anchoring the M proteins and causing the pr protein to be absent in the mature viral membrane (Pokidysheva, Zhang et al.2008)[13,14].

Figure 1: Dengue Virus Genomic Structure

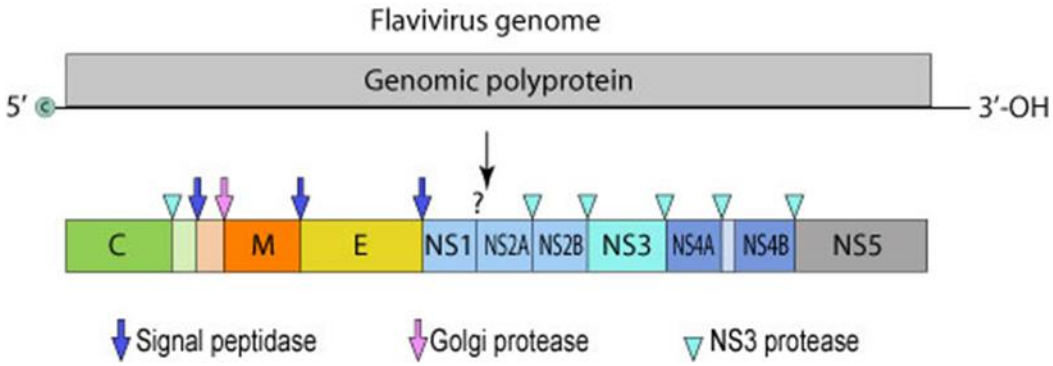
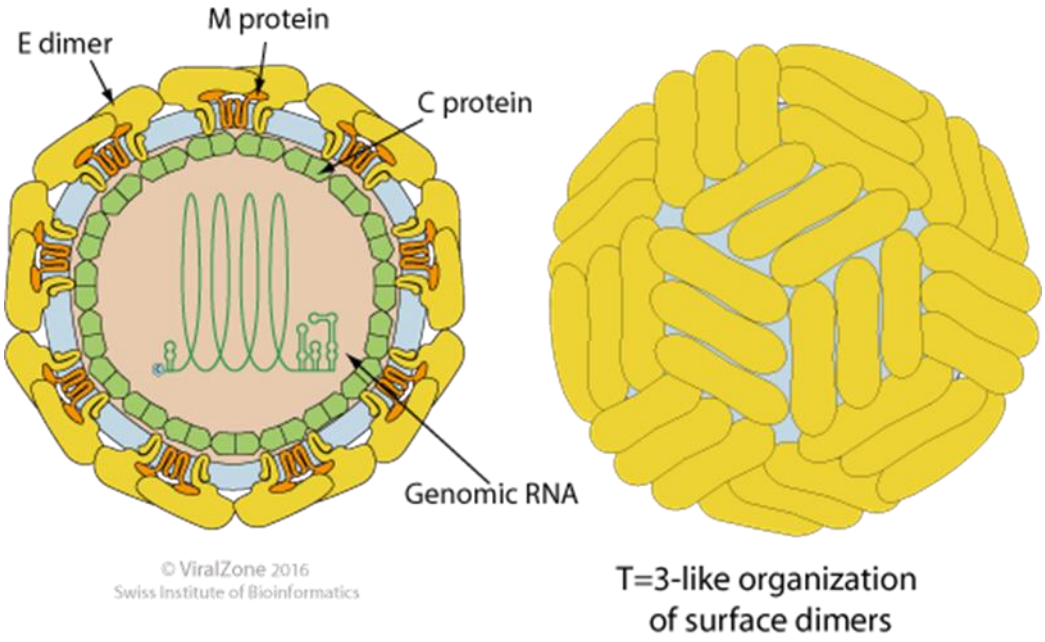


Figure 2: Morphology of Dengue Virus



PATHOGENESIS OF DENGUE:

The disease manifest as in apparent clinical symptoms to severe manifestation like DHF/DSS due to multifactorial reasons. After the bite of infected female Aedes Aegypti/albopictus , the infected individuals develop disease varying from 3 to12 days .These mainly depends on two major factors such as viral and host factors .Viral factors are viral virulence, viral tropism and antibody dependant enhancement. Host factors are age, sex, race, nutritional status, genetic factors, complement activation ,transient autoimmunity ,Cross reactive T cell response and Soluble factors.

The virus inoculated into epidermal dendritic cells (DC)/immature Langerhans cells and keratinocytes which migrates to local lymph nodes. Monocytes and macrophages are directed at these sites. The infected monocytes and macrophages will enter into circulation resulting in primary viremia which causes infection in several cell lines of mononuclear lineage including blood derived monocytes, myeloid DC, splenic and liver macrophages[17,18].

During secondary infection with heterologous DENV infection, high concentration of specific immunoglobulin's will combine with newly produced virus that adhere to and taken up by the mononuclear cells. Following infection, these cells predominantly die by apoptosis and more viruses released into circulations. However partially infected/bystander DC are stimulated which produces bulk of inflammatory mediators and haemostatic response of the host[19,20].

In spite of affecting several organs like spleen, liver, kidney, lungs and brain there is no microscopic evidence of organ pathology. It affects liver without causing morphological damage. Liver cells may die by apoptosis without any inflammation[21]. Endothelial cells (EC) have an important role in coagulation response with severe systemic Dengue infection.EC do not carry FCγR on their surface so it will not take up antibody coated virus. However lung and abdominal vasculature may contain virus by pinocytosis[22] .Infection in these cells produces no morphological damage, only causes functional damage.NS1binds to EC of pulmonary and hepatic tissues .Selective apoptosis of micro vasculature in lung and intestinal tissues leads to plasma leakage and fluid collection seen in pleural and peritoneal cavity[23].

DENV has 5 serotypes (ST) and many genotypes. Viral genetic difference is associated with differences in virulence. It evolves during an epidemic into a severe form of strain by continuous passage. High incidence of severe Dengue infection is seen in secondary infections with DENV-2 and DENV-3 following DENV -1 infection. The incidence of disease severity increases if longer the interval between primary and secondary infection. Severe infections in children are observed in Asia but in America adult population are mostly affected. Black people have more protection than white people. Males are more affected than females and children[24,25].

Activation of complement is an important role in pathogenesis of Dengue. Complement is activated by binding of heterotypic antibody to NS1 antigen which is expressed on infected cells.C5b-C9 complex stimulate the production of inflammatory

cytokines responsible for activation of coagulation system which is associated with DHF/DSS. Coagulation enzymes and IgG1, IgG3 also effectively activates the complement system[25,26].

In transient autoimmunity cross reaction of anti NS1 antibody with cells of liver, platelets and EC is observed. NO (nitric oxide) is produced from EC which undergo apoptosis and leads to plasma leakage.NO production inhibit DENV replication and its over production leads to cell damage[27]. Transient thrombocytopenia caused by anti NS1 antibodies cross react with platelets. So there was a controversy in production of live attenuated vaccines. These antibodies also enhances expression of I-L6, IL-8 and ICAM-1(intra cellular adhesion molecule)[28].

In cross reactive T cell response,CD8+ T cells with high avidity for infecting virus secretes high concentration of pro and anti-inflammatory cytokines such as IFNγ, TNFα and IL-13 but low levels of IL-10. These high avidity cross reactive CD8+ Tcells die by apoptosis. Alternatively low avidity CD8+ T cells for heterologous virus expand to produce high level of pro inflammatory cytokines but lose their cytolytic activity. Due to increased frequency and higher activation state cross-reactive memory T cells for the primary infecting virus are effectively activated. This phenomenon is called “Original antigenic sin”(OAS).It is based on TCR(Tcell receptor) variation in different individuals[29,30].

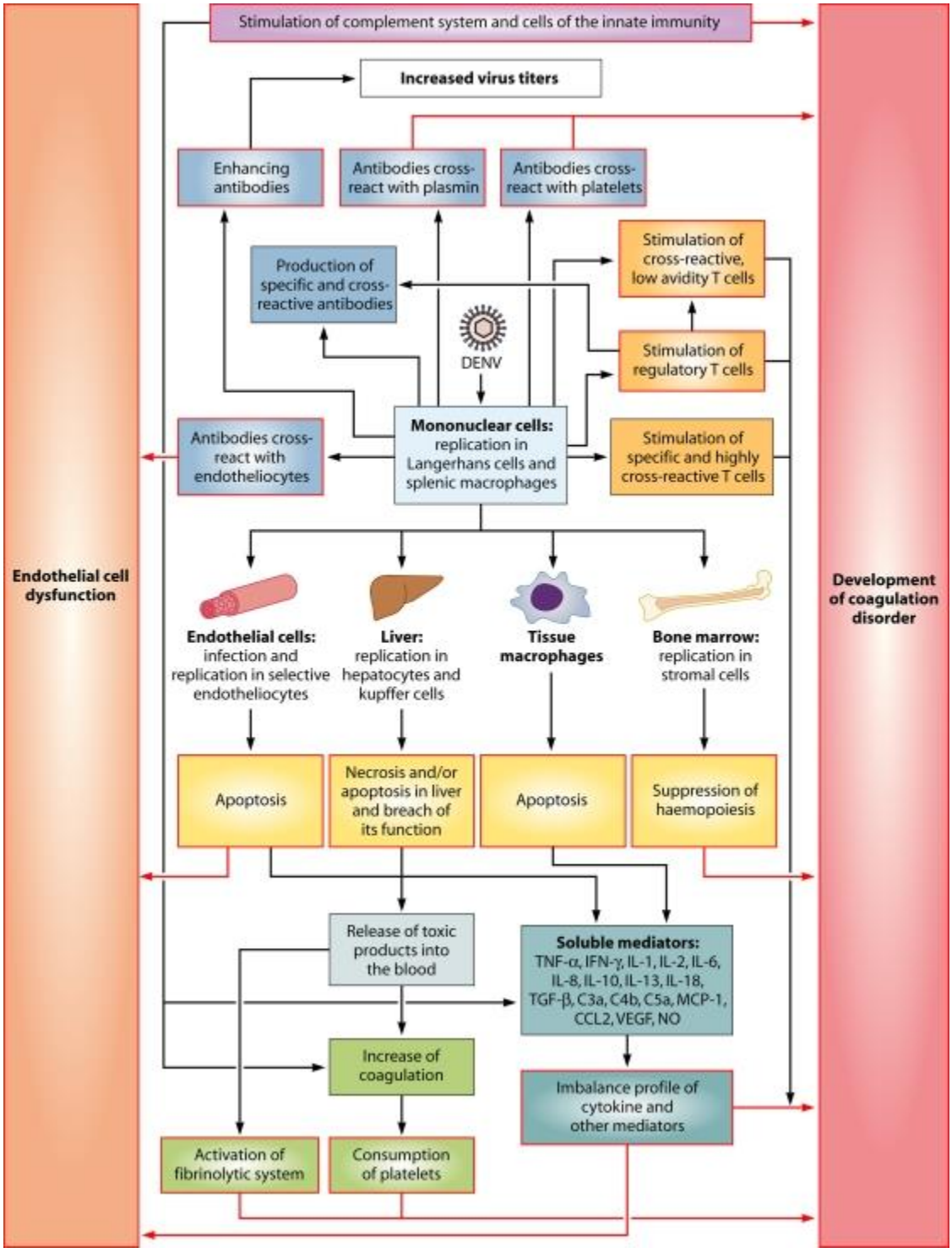
Host genetic factors constitute an important component in the susceptibility of disease in both individual and population level. DHF is associated with several HLA class I, II alleles and polymorphism in TNFα, FcγR,Vitamin D receptor,CTLA-4,DC-SIGN and TGFβ. Individuals with G6PD deficiency have decreased NO,H2O2 and super oxide production which can increase viral proliferation and viral virulence. MBL-2 leads to thrombocytopenia in DHF[31].

Soluble factors are associated with severe DHF/DSS. A "storm" of inflammatory cytokines and other mediators are secreted by an increase viral load and non protective T cells in host leading to increased plasma leakage characteristic of DHF/DSS.Increased levels of IL-1β, IL-2, IL-4, IL-6, IL-7, IL-8,IL-10, IL-13, IL-18, TGF-α, TNF-1β , and IFN-γ are seen in severe disease[32,33]. Multiple cytokines contribute simultaneously in a complex way to the development of DHF/DSS. DSS patients recover rapidly as cytokines do not cause tissue destruction and reversal of EC dysfunction.

The soluble factor Thrombin converts fibrinogen to fibrin and triggers platelet activation .These activated platelets release a soluble factor called MMP9 which enhances easy permeability leading to plasma leakage and edema[34]. Thrombin also activates complement factors such asC3a and C5a enhancing blood thrombogenicity .Thrombin stimulates the production of IL-10 by monocytes. This IL10 inhibits expression of Tissue factor (TF) fibrinolysis. IL-6 is a potent inducer of fever .Due to EC damage in the liver, IL8 is also abundantly produced . IL-8 induces the expression of adhesion molecules ICAM-1 and VCAM-1. TNF-α enhances capillary permeability and

activates the fibrinolytic system .It enhances the expression of NO which induces apoptosis of cells and also enhances vasodilatation leading to edema formation. TNF-α mediates death of T cells leading to peripheral T cell deletion .TGF-β, late in the infection ,enhances the production of Th2 cytokines such as IL-10 and inhibits Th1response.Activation of vascular endothelial growth factor (VEGF) will also increases vascular permeability[35].

Figure 3: Pathophysiology of Dengue



BIOMARKERS FOR PREDICTION OF DENGUE SEVERITY:

Biomarkers help in the prediction of severe Dengue in patients without any warning signs. An ideal biomarker should be able to identify individuals who are at risk of developing severe Dengue. There are four types of biomarkers

- Immune activation markers
- Endothelial activation markers
- Biochemical markers
- Genetic markers

The advantages of these biomarkers include accuracy and helps in providing early supportive care. The disadvantages include cost, requirement of sophisticated instruments and altered levels may be seen in other diseases also[36].

Table1: Biomarkers of severe Dengue disease

Class	**Biomarker**		**Change**
1. Immune activation markers	Cells	Plasmacytoid dendritic cells	Decrease
		Lymphocytes	Decrease
		Platelets	Decrease
	Cytokines	IL-10	Increase
		MIF	Increase

Class	Biomarker		Change
	Chemokines	CXCL-10	Increase
	Complements	C3a, C5a	Increase
	Soluble receptors	sCD4/8, sIL-2R, sTNFRII	Increase
	Proteases	Tryptase and chymase	Increase
2. Endothelial activation markers	Mediators of endothelial function	Angiopoietin-1	Decrease
		Angiopoietin-2	Increase
	Coagulation pathway components	von Willebrand factor	Increase
		ADAMTS-13	Decrease
		sThrombomodulin	Increase
	Cell surface adhesion molecules	sICAM, sVCAM	Increase
	Permeability mediators	VEGF, VEGFRI	Increase
		VEGFRII	Decrease
3. Biochemical markers	Lipids	Total cholesterol, HDL, LDL	Decrease
	LPS	LPB, CD14	Increase

Class	Biomarker		Change
	Liver enzymes	AST, ALT	Increase
	Serine protease	IaIp	Decrease
	Other soluble substances	Nitric oxide	Decrease
4. Genetic markers	Gene profile	Certain gene expression	Decrease
	Circulating cell free-DNA		Increase

DIFFERENTIAL DIAGNOSIS:

The differential diagnosis of Dengue fever varies by country, region, season specific and includes Measles, Rubella, Enterovirus, Influenza, Typhoid, Chikungunya, Scarlet fever, Malaria, Leptospirosis, Hepatitis A, Rickettsiosis, Bacterial sepsis, Other viral hemorrhagic fevers- Ebola, Lassa fever, West nile fever, Rift valley fever.

EPIDEMIOLOGY OF DENGUE:

SEROTYPES:

There are four closely related but serologically distinct Dengue viruses, named DENV-1, DENV-2, DENV-3 and DENV-4, belongs to the genus Flavivirus. The fifth and the latest serotype have been declared in October 2013, called DENV-5. This serotype was found in the sample of a 37 year old farmer from Sarawak state of Malaysia

in 2007[79]. The first four serotypes exhibit transient and weak cross protection among themselves, therefore in an endemic area individuals can be infected with up to four serotypes in a lifetime. There may be hyperendemicity in a region leading to periodic epidemics. Initially DENV-5 was thought to be a variant of Dengue 4 serotype, but infection in the rhesus monkeys that were previously infected with other four serotypes leads to significantly different set of antibodies. This proved that the new virus was indeed a new serotype and not a variant of DENV-4[15,16].

DENGUE IN INDIA:

Dengue viruses have been persisting in India year after year since 1956. In Tamilnadu, the first major outbreak of Dengue was noticed in Vellore, South Arcot district in 1961 and the viral etiology was established later by the isolation of Dengue virus (Carey et al., 1966). The first virologically proved epidemic of Dengue fever in India occurred in Calcutta and eastern coast of India in 1963 - 1964 (Sarkar et al., 1964; Chatterjee et al., 1965; Carey et al., 1966). Then, the Dengue infection spread northwards and reached Delhi in 1967 (Balaya et al., 1969). Subsequently, the whole country was involved with widespread epidemics followed by endemic or hyper endemic prevalence of all four serotypes of Dengue virus. After the occurrence of first epidemic in 1961, Vellore experienced outbreaks in the year 1964, 1966 and 1968. The virological investigations carried out during that period proved the presence of Dengue 2 in 1964 outbreak (De Ranitz et al., 1965; Carey et al., 1969). Dengue 3 virus was isolated in 1966 outbreak (Myer et al., 1969) and all four types of Dengue virus in 1968 outbreak (Myer et

al., 1970). The epidemic at Vishakapatnam in 1964 was due to Dengue 2 virus (Krishnamurthy et al., 1965; Paul et al., 1965). The epidemic of Dengue in Nagpur in 1965 documented the presence of Dengue 4 virus in that region (Rodrigues et al., 1972). In the same year, another outbreak was observed in Madras(now Chennai) which was caused by Dengue 3 viruses (Myers et al., 1968). Later, outbreaks of Dengue occurred in Jabalpur (MP) by Dengue virus 3 in 1966 (Sehgal et al., 1967, Rodrigues et al., 1973) in Asansol in 1967 by Dengue 2 and 4, in Delhi in 1967 by Dengue 2 (Balya et al., 1969), in Kanpur in 1968 and 1969 by Dengue 4 and Dengue 2 (Chaturvedi et al.,1970 ; Chaturvedi et al.,1972), in Ajmeer in 1969 by Dengue 1 and Dengue 3 (Ghosh et al., 1974), in 12 Gwalior in 1970 by Dengue 3 (Arora et al., 1970), in Bangalore in 1971 by Dengue 1 and Dengue 2 (George and Soman, 1975); (Raghavan et al.,1970) in Jaipur in 1971 and 1973 by Dengue 1 and 2 (Padbiri et al., 1973; Mathew et al., 1976), in Jammu in 1974 by Dengue 2 (Mathew et al., 1977) and in Trichur in 1974 by Dengue 2 (Sreenivasan et al.,1979). Dengue 3 has been isolated during the epidemic at Calcutta in 1983 (Mukerjee et al., 1987). An epidemic of Dengue at Rajasthan in 1985 was due to Dengue 3 virus (Chouhan et al., 1990). Dengue 2 was isolated during the epidemics of Dengue in urban and rural areas of Gujarat state during 1988 and 1989 (Mahadev et al., 1993). Outbreaks occurred at Gwalior in 2003 and 2004 by Dengue 3 (Dash et al., 2005, 2006). Padbiri et al., (1995) reported Dengue in Mangalore, Karnataka in 1993. In Punjab, there was an outbreak of Dengue in 1996 (Kuldip et al., 1997). The outbreak of Dengue in Delhi in 1996 was due to Dengue 2 (Chusak et al., 1993; Dar et al., 1999).

Hence, the presence of all four types of Dengue virus and occurrence of the disease all over the India were well documented.

DENGUE IN TAMILNADU:

As stated earlier, in Tamilnadu, the outbreaks of Dengue were noticed in Vellore, North Arcot district in 1961 (Carey et al., 1964, 1966 and 1968; Carey et al., 1969; Myers et al., 1970). During this period, an outbreak was also noticed in Madras (Chennai) in 1965 (Myers et al., 1968). The occurrence of Dengue fever was reported in villages in Dharmapuri district, Tamilnadu in 1997 (Kader et al., 1997) and in 2001 (Victor et al., 2002). The presence of Dengue fever for the first time in Coimbatore and Erode district of Tamilnadu was reported during 1998 by Singh et al .,(2000). There was an outbreak of Dengue in Chennai in 2001 which was caused by Dengue 2 and Dengue 3 viruses. (Kabilan et al., 2005). The outbreak of Dengue in Kanyakumari district in July 2003 proved the presence of Dengue serotype 3 virus in that area (Paramasivam et al., 2006).

CLINICAL FEATURES OF DENGUE INFECTION:

The typical clinical manifestation of Dengue infection range from self limiting Dengue fever to Dengue haemorrhagic fever with shock syndrome.

ASYMPTOMATIC INFECTION:

Most of the Dengue virus infections in adults are symptomatic as compared to children under the age of 15 who are asymptomatic or minimally symptomatic[37].

CLASSICAL DENGUE FEVER:

It is an acute febrile illness characterized by headache, retro orbital pain and severe muscle, joint pains (break bone fever). Clinical symptoms usually begin between 4 and 7 days after the mosquito bite. The incubation period may range from 3 to 14 days. Fever classically lasts for five to seven days, with a biphasic curve in some patients (saddleback), with the second febrile phase lasting for one to two days. Febrile phase may be followed by a phase of marked fatigue that can last for days to weeks. Symptoms have age and sex predilection. All symptoms were less frequent in patients less than 19 years of age. Females have joint pain, body aches and rashes. Rashes were commonly seen during primary infection, whereas constitutional and gastrointestinal symptoms were common in secondary infection. Rashes were seen 2 to 5 days after the onset of fever[38,39,40].

HAEMORRHAGIC MANIFESTATIONS:

Haemorrhagic manifestations occur commonly in Dengue and can be life threatening rarely. The common bleeding sites are skin, nose and gastrointestinal system in descending order[41].

OTHER SYMPTOMS:

a) Constitutional symptoms, including fever
b) Headache, retro orbital pain, body ache, and joint pain
c) Rash
d) Nausea, vomiting and diarrhoea

e) Cough, sore throat, nasal congestion[42].

Table 2: Common Clinical Features of Dengue (WHO:2009)

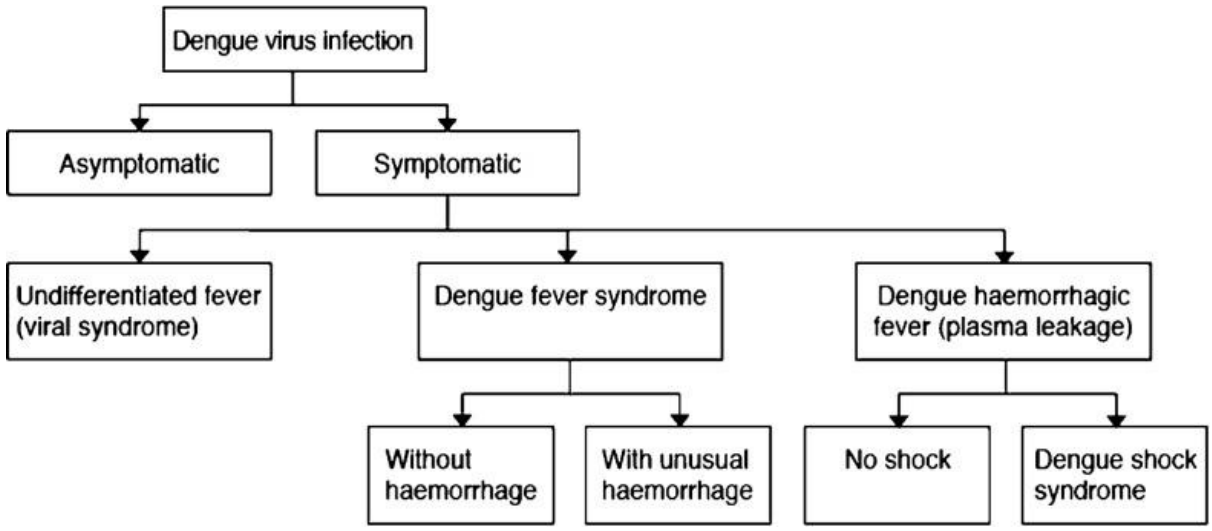
Clinical Symptoms	Number of Cases (%)
Fever	100
Thrombocytopenia	77
Maculopapular Rash	31
Elevated Liver Enzymes	51
Vomiting	35
Headache	26
Arthralgia/ Myalgia	32
Bleeding Manifestation	25
Pain Abdomen	18
Conjucntival Congestion	10
Respiratory Complaints	11
Neurological Complaints	10
Hypotension	8
Diarrhoea	9

WHO CLASSIFICATION OF DENGUE:

There are two classification systems for Dengue infection depending on the clinical status. They are called Old (1997) classification and New (2009) classification.

OLD CLASSIFICATION (1997):

Figure 4: Old Dengue Classification



NEW CLASSIFICATION (2009):

The WHO special program for research and training in tropical diseases and the Pan-American health organization proposed a revised classification system in 2009[43]. Based on this classification there are two broad categories:

1) Non severe Dengue
2) Severe Dengue

Non severe Dengue can be further divided into Dengue with or without ‘warning signs’ which includes

a) Abdominal pain or tenderness
b) Persistent vomiting
c) Clinical signs of fluid accumulation
d) Mucosal bleeding
e) Lethargy or restlessness
f) Liver enlargement >2cm
g) Increase in haematocrit concurrent with rapid decrease in platelet count

The term ‘Severe Dengue’ can be applied to the patients with following features

a) Severe plasma leakage- leading to shock or fluid accumulation with respiratory distress
b) Severe haemorrhage (as defined by the treating physician)
c) Severe organ impairment (AST or ALT>1000, impaired consciousness, severe involvement of heart or other organs)

PHYSICAL EXAMINATION:

Conjunctival injection, pharyngeal erythema, lymphadenopathy, hepatomegaly, facial puffiness and palatal petechiae. Rash is typically macular or maculopapular and may be associated with pruritis[44].

LABORATORY FINDINGS:

a) Leukopenia- common in children and adults
b) Thrombocytopenia – platelet<100,000 cells/cu.mm
c) Elevated serum aspartate transaminase- both in adults and children[45].

DENGUE HAEMORRHAGIC FEVER:

It is the most dreadful complication of Dengue fever and is associated with circulatory failure and shock. The four cardinal features are

a) Increased vascular permeability- plasma leakage syndrome- characterized by hemoconcentration (20% or greater rise in haematocrit above the baseline value), pleural effusion or ascites.
b) Marked thrombocytopenia- platelet<100000cells/cu.mm
c) Fever lasting for 2 to 7 days.
d) Haemorrhagic tendency or spontaneous bleeding[45].

When these features are associated with shock, the term Dengue shock syndrome is used.

PLASMA LEAKAGE:

It is the most specific and life threatening feature of Dengue haemorrhagic fever. Increased vascular permeability can develop over a period of 24 to 48 hours. Marked plasma leakage leads to shock in case of delayed supportive measures. It usually occurs between 3 to 7 days after the onset of illness. This will coincide with defervescence, severe thrombocytopenia and elevated aminotransferases. Abdominal pain, persistent

vomiting, marked restlessness or lethargy and coincidence with defervescence should raise the suspicion of impending DSS. Imaging is needed for detection of pleural effusion and ascites[45,46].

HAEMORRHAGIC MANIFESTATIONS:

The features include spontaneous petechiae or ecchymoses, hematemesis, malena, menorrhagia and epistaxis[46]. Positive tourniquet test demonstrates microvascular fragility. This is done by inflating a blood pressure cuff on the arm to midway between systolic and diastolic blood pressures for five minutes. The pressure is released for at least one minute and the skin below the cuff is examined for petechiae. A finding of 10 or more petechiae in a one square inch area is considered positive.

OTHER MANIFESTATIONS:

Liver failure may be caused by prolonged hypo perfusion or hypoxia rather than a direct viral effect.

Neurological manifestations include encephalopathy and seizures. Symptoms include fever, headache and lethargy. Other associated neurological syndromes are acute pure motor weakness, mononeuropathies, polyneuropathies, Guillain-Barre syndrome and transverse myelitis. Reye syndrome has been seen in children associated with use of salicylate medications rather than Dengue infection per se[47].

Myocardial dysfunction has been seen in hospitalized patients. It may be due to the fluid overload while treating shock. Elevated Troponin-I or the N terminal fragment of B-type natriuretic peptide may be seen.

Acute kidney injury has been reported. It may be due to the complication of shock, or due to rhabdomyolysis, glomerulonephritis and acute tubular necrosis[48].

Other rare manifestations include cholecystitis and retinal vasculitis. Hemophagocytic lymphohistiocytosis may be associated with Dengue fever[49].

Based on these data it was concluded that the vaccine may yield a public health benefit, though the durability of vaccine impact and level of herd immunity required to affect Dengue transmission are both unknown. According to WHO strategic advisory group of experts the vaccine should be introduced only in geographic settings with seroprevalence> 70% in the target age group. It should not be used if the seroprevalence is <50%.

LABORATORY DIAGNOSIS:

The laboratory diagnosis of Dengue infection is based on the following Direct and indirect methods. Directs methods are detection of RNA in serum or tissues, virus isolation and detection of viral antigens(NS1).Indirect methods are detection of specific antibodies in serum(IgM & IgG).If the test has high accessibility, the confidence interval is low(Indirect methods) but if the test has low accessibility the confidence interval is high(Direct methods).

VIRUS ISOLATION:

The following methods can be used for isolation of Dengue viruses by intracerebral inoculation of new born mice, Inoculation on mammalian cell cultures, intrathoracic inoculation of adult mosquitoes and Inoculation on mosquito cell cultures[50,51].

INTRACEREBRAL INOCULATION:

All the four serotypes can be isolated by intracerebral inoculation of suckling mice[52,53,54]. The limitations are high cost, long time for isolation and low sensitivity.

MAMMALIAN CELL CULTURE:

In this culture, the viruses frequently require multiple passages before inducing cytopathic effects in infected cells. They also have limitations like that of intracerebral inoculation, hence not routinely recommended for virus isolation[51]. LLCMK2(monkey kidney), Vero(monkey kidney), BHK21(baby hamster kidney) are examples of mammalian cell lines.

MOSQUITO INOCULATION:

It is the most sensitive but least used method for virus isolation[55]. The mosquito species used are Aedes Aegypti, A.albopictus ,Toxorhynchitiesamboinensis and T.splendens. Both male and female mosquitoes can be used. Mosquito tissues like brain, salivary glands and intrathoracic inoculation can be used. DengueDengue viruses replicate in high titres within four to five days after appropriate incubation and detected

by immunofluorescence assay (IFA). The limitations are need for insectaries to produce large number of mosquitoes, hard work and isolation precautions to prevent the release of infected mosquitoes[56,57].

MOSQUITO CELL CULTURE:

It is the latest method for virus isolation. The most widely used cell lines are Aedes Aegypti, A.albopictus(C6/36) , Toxorhynchities amboinensis(Tra-284) and T.splendens, A.pseudoscutellaris(AP61,AP64 and CLA-1) .The Dengue antigens in the infected cell culture can be detected by immunofluorescence assay. Advantage is rapid, sensitive and economical. It is more sensitive than vertebrate culture system, easy to maintain and grow at room temperature, can kept for 14 days without a change of growth medium and carried into the field and inoculated directly with human serum[58,59,60].

NUCLEIC ACID DETECTION:

These methods can readily detect Dengue viruses during the acute phase and sometimes during the convalescence phase. The methods used are

- Nucleic acid hybridization
- Reverse transcription- polymerase chain reaction

NUCLEIC ACID HYBRIDIZATION:

In nucleic acid hybridization, the RNA extracted either from Dengue virus infected cell culture supernatants or pools of infected A.albopticus are hybridized either with biotinylated probes or 32P- labelled probes[61]. Methods using biotinylated probes are less sensitive than radiolabelled probes. It is primarily used in epidemiological studies.

RNA-RNA hybridization is a sensitive method that can be applied directly on fresh samples or on retrospective analyses of fixed samples. It is more often used as a research tool[62,63].

RT PCR:

It offers better sensitivity than virus isolation with a rapid turnaround time. Best clinical sample for RT-PCR is serum/plasma while compared to other human clinical samples, biopsies, autopsy tissues or mosquitoes. It involves three basic steps nucleic acid extraction and purification, amplification of the nucleic acid, detection and characterization of the amplified product.

Extraction and purification of viral RNA from the specimen can be done by QIA amp Viral RNA kit(automated magnetic bead based) or by a liquid phase separation methods (phenol/ chloroform), or by solid silica based commercial kits (column based).Its results are based on region of the genome amplified and primer are used to achieve this goal. Lanciotti et al developed consensual primers (D1&D2) which amplify at 511bp fragment of C and prM genes of Dengue in conventional RT-PCR, after that agarose gel electrophoresis is done to identify the amplified product of 511bp fragment to

confirm DENV infection. The sensitivity of this test varies from 80 to 100%. False positives can occur as a result of contamination by amplicons from previous amplifications[64,65,66].

NESTED PCR:

Detection of Dengue virus is three to four fold increased by using nested-PCR.It can be type specific for Dengue serotyping (DENV1-4) with detection threshold of less than 100 PFU. In nested PCR assay, universal Dengue primers are used targeting the C/prM region of the genome. Double-stranded DNA product of the RT-PCR was typed by a second round of PCR amplification (nested PCR) with type-specific primers, which yielded DNA products, the unique sizes of which were diagnostic for each Dengue virus serotype (TS1-482bp, TS2-119bp, TS3-290bp, TS4-392bp).This amplified products are identified by using agarose gel electrophoresis[65].

ONE-STEP MULTIPLEX PCR:

It is an alternative to nested PCR in which there is a combination of four serotype specific oligonucleotide primers in a single reaction tube. The products of these reactions are separated by agarose gel electrophoresis and the amplification products are visualized as bands of different molecular in the gel using ethidium bromide dye[67].

REAL-TIME RT PCR:

It is a one-step assay system for quantification of viral RNA using primer pairs and probes that are specific to each Dengue serotype. Fluorescent probe enables the detection of reaction products in real time in a specialized PCR machine. The assays that have been developed are TaqMan or SYBR Green technologies. Off which TaqMan assay is highly specific due to the sequence specific hybridization of the probe[67].

Real-time RT PCR assays can be either singleplex or multiplex. The multiplex assays can detect all four serotypes in a single reaction without introduction of contamination. Advantages are rapidity, ability to provide quantitative measurements, lower contamination rate, easy to standardize, higher sensitivity and specificity. They are less sensitive than nested PCR assays[67].

NASBA:

Nucleic acid sequence based amplification assay is an isothermal (41˚C) RNA specific amplification that does not require thermal cycling instrumentation. The first step is a reverse transcription in which the single stranded RNA target is copied into a double stranded DNA molecule that serves as a template for RNA transcription. Detection of amplified RNA is done by electrochemiluminescence or in a real time by fluorescent labelled molecular beacon probes. It has sensitivity as that of virus isolation[68].

RT-LAMP:

This loop mediated isothermal amplification assay detects 3'noncoding region of DENV serotypes. It is simple, rapid and amplification can be obtained in 30 minutes at isothermal condition (63˚C).

NUCLEOTIDE SEQUENCING AND PHYLOGENETIC ANALYSIS:

Nucleotide sequencing of the C-prM gene junction of DENV is carried out by Big Dye Terminator Cycle Sequencing Ready Reaction Kit with ABI 3100 sequencer (Applied Biosystem, USA) for identifying genotype of DENV serotypes by standard protocol. The sequence is entered into BLAST to identify the close sequence. By using Neighbour Joining (NJ) method of MEGA3 software version, the phylogenetic analysis is done based on C-prM gene junction of serotypes by including a large number of geographically diverse DENV gene sequences. It is mainly used in outbreak to identify origin of serotypes by using phylogenetic tree generated by NJ method[64].

DETECTION OF NS1 ANTIGEN:

NS1 is a 50kDa glycoprotein synthesized by all flaviviruses. During acute phase of infection, NS1 protein is secreted by Dengue virus and found associated with intracellular organelles or it is transported through the cellular secretory pathway to the cell surface. This hexameric form is released from infected mammalian cells but not from vector derived mosquito cells. Presence of high concentrations of hexameric form of NS1 antigen indicates impending risk of severe Dengue.it is detectable from day 1 to day 18 but peak at day 1 to7.

Dengue NS1 Ag MICROLISA is an in vitro test for detection of NS1antigen in serum or plasma. It is a solid phase ELISA based on the direct sandwich principle. Only human serum or plasma should be used. Specimens should be free of microbial contamination and stored at 2 to 8˚C for one week or can be frozen at -20’C. Repeated freezing and thawing should be avoided.

The micro wells used in this test are coated with anti-Dengue NS1 antibodies with high reactivity for Dengue NS1 antigen. The samples are added to the wells followed by addition of enzyme conjugate (monoclonal anti Dengue NS1antibodies linked to horseradish peroxidase). A sandwich complex is formed in the well wherein Dengue NS1 is trapped or sandwiched between the antibody and antibody HRPO conjugate. Unbound conjugate is washed off with wash buffer. The amount of bound peroxidase is proportional to the concentration of NS1 antigen present in the sample. Addition of the substrate buffer and chromogen leads to development of blue colour. The intensity of the colour is proportional to the concentration of antigen in the sample. To limit the enzyme-substrate reaction, stop solution is added and a yellow colour develops which is read at 450nm spectrophotometrically.

If the Dengue NS1 Ag units is <9, then the sample is negative for Dengue. If between 9 to 11 units, then the test in equivocal. If >11 units, the sample is positive for NS1 antigen. The sensitivity of this test is 99.5% and specificity is 100%.

Limitations of this test are, used for NS1 antigen detection only in serum or plasma, it's a screening test only and false positive results can occur due to its cross reactivity[69].

SEROLOGICAL DIAGNOSIS:

The serological tests used for the diagnosis of Dengue infection includes

- Haemagglutination inhibition(HAI),
- Complement fixation Test (CFT),
- Plaque reduction Neutralization test(PRNT),
- Indirect immunofluorescent-antibody test
- Immunoglobulin M (IgM) capture enzyme linked immunosorbent assay (MAC-ELISA),
- Indirect immunoglobulin G(IgG) capture ELISA,
- Dot blotting,
- Western blotting,
- Rapid immunochromatography test

HAEMAGGLUTINATION INHIBITION (HAI):

This test was introduced by Sabin and colleagues and modified by Casals and Brown. It was used as a standard diagnostic method for the diagnosis of Dengue due to its high sensitivity and ease of use. It was used in seroepidemiological studies and to differentiate primary and secondary Dengue infections. Dengue viruses agglutinate gander erythrocytes and trypsinized type O human red blood cells. The HI test is based

on the ability of the Dengue virus antibodies to inhibit this agglutination. The procedure is carried out in the following steps.

- Sera should be extracted with kaolin or acetone and adsorbed with gander or trypsinized type O red blood cells
- All wells in micro titre plate receive 0.025ml containing 8-16 HA units of each Dengue antigen. Plates are covered and incubated at 4 degree C overnight.
- Next morning plates are kept in room temperature, 0.05ml of an 8% goose red cell stock solution diluted 1:24 in the proper pH buffer is added to each well. Plates are observed for one hour and agglutination scored.
- All sera from a single patient should be tested in the same assay. As a screening test a single antigen may be used (DENV-1 or 4) with little loss of sensitivity in primary infections. If the screening test for paired sera is negative, the specimens may be retested against all Dengue antigens. Known positive and negative sera should be included in each test to standardize the results and to maintain quality control.

In primary infections the antibodies are detected from fifth or sixth day of symptoms when the titres are above 1:10. The antibody titres are also low in convalescent sera (1:640) in primary infections. On the other hand the antibodies are detected very early in secondary or tertiary infections, usually higher than 5:120. Thus, a higher titre of antibodies (1:1280) during the acute phase or at the beginning of convalescent phase of disease is an indication of secondary infection[70]. The

limitations of this test are its lack of specificity, need for paired samples and inability to identify the serotype.

Table 3: WHO criteria for interpreting HI assay results

Antibody response	Time period between acute and convalescent samples	Convalescent titre	Interpretation
≥4-fold rise	≥7 days	≤1:1280	Acute flavivirus infection, primary
≥4-fold rise	Any specimen	≥1:2560	Acute flavivirus infection, secondary
≥4-fold rise	<7 days	≤1:1280	Acute flavivirus infection, either primary or secondary
No change	Any specimen	>1:2560	Recent flavivirus infection, , secondary
No change	≥7 days	≤1:1280	Not dengue
No change	<7 days	≤1:1280	Uninterpretable
Unknown	Single specimen	≤1:1280	Uninterpretable

COMPLEMENT FIXATION TEST:

This test is not routinely used for the diagnosis of Dengue infection. It is based on the principle that complement will be consumed during antigen-antibody reaction. The antibodies usually appear later than HI antibodies and also persist for short periods and hence they have limited value in seroepidemiological studies. They are very specific in primary infections and contributes to the identification of infecting serotype. The limitations of this study are requirement of trained personnel and difficult to perform[71,72].

PLAQUE REDUCTION NEUTRALIZATION TEST

This test was developed by Halstead, Nisalak and Sukhavachana. This assay was developed to measure the DENV neutralizing antibody and uses “probit analysis” to measure plaque reduction at a 50% endpoint (PRNT50). It is the gold standard test for measuring Dengue antibodies.

Few variations in this technique were introduced, such as a micro metabolic inhibition test using BHK-21 cells, micro culture plaque reduction test utilizing the LLC-MK2 cell line, BHK-21 cells, a focus reduction method using peroxidase-antiperoxidase staining of BHK-21 cells, a screening test using a single dilution and a 70% plaque reduction endpoint and a simplified PRNT assay using BHK-21 cells.

It is necessary to select the cell line capable of supporting plaque formation with each of four Dengue viruses, preferably LLC-MK2, Vero and BHK-2 cells.

The PRNT involves incubating a constant number of plaque forming units (PFU). The standard protocol uses LLC-MK2 cells in six-well culture plates. Serial fourfold test serum dilutions, positive and negative serum controls are added to an equal volume of virus suspension diluted to contain 50 PFU’s in the inoculum volume, placed in a shaker and incubated at 37’C for one hour. The mixtures are then placed in an ice-bath, inoculated onto cells and allowed to absorb onto cells for one hour at room temperature. Then the first overlay medium containing an agar-nutrient mixture is added and incubated at 37˚C for 7 days, after which a second overlay containing neutral red is added, incubated at 35˚C overnight. Plaques are counted in each well on a fluorescent light box.

Use of higher percent plaque reduction endpoints will increase the specificity but decreases the sensitivity. No standards have been adopted for interpreting the assay. Earlier in the infection the neutralizing antibodies are of IgM type fairly specific to the infecting type. Later in the infection the antibodies are IgG.

The limitations of this assay are its high cost, time consuming and technical difficulties[70].

MAC-ELISA:

It is considered as a useful test for the diagnosis of Dengue. Serum, blood on filter paper, saliva and CSF (Dengue encephalitis) can be used for detection of IgM if samples are taken within the appropriate time (five days or more within the onset of fever). Serum specimens can be tested at a single or multiple dilutions. Most of the antigens used for this assay are derived from Dengue virus envelope protein.

Total IgM is captured by anti μ chain specific antibodies coated on a micro plate. Dengue specific antigens (DENV 1 to 4 serotypes) are bound to the captured anti-Dengue IgM antibodies and are detected by monoclonal or polyclonal antibodies directly or indirectly conjugated with an enzyme that will transform a non-coloured substrate into coloured products. The optical density is measured by spectrophotometer (18). Results interpretation by using PANBIO IgM CAPTURE ELISA positive results >11panbio units (1.1 index), negative <9 panbiounits(0.9 index) and equivocal means 9-11 panbio units(0.9-1.1 index)[70].

Figure 4 Principle of MAC-ELISA test. Sensitivity of this test is much less than HI in acute phase samples, but the specificity is similar. It has been used for the surveillance of Dengue and DHF/DSS. It has the advantage of rapid diagnosis during epidemics and no inhibitor in patient's serum so no need of pre-treatment of test sera. The limitations of this test are cross reactivity with other flaviviruses[72,73].

IgG ELISA:

It is useful for the detection of recent or past Dengue infection when paired sera are collected within the correct frame of time. The assay uses same antigens as in MAC-ELISA[70]. Use of E/M specific capture IgG ELISA (GAC) helps in detection of IgG antibodies (peak at 6-15 days following infection)over a period of 10 months after the infection. It is used for the differentiation of primary and secondary infections and seroepidemiological studies. HAI titres> 1:2560 is Pnabio IgG cut off value is set to detect high levels of IgG antibodies above this threshold. It will not detect low level of IgG in many individual from endemic.so high level indicative of active secondary DENV infection. Results interpretation by using Panbio IgG Capture ELISA positive results >22panbio units (2.2 index), negative <18 panbiounits (1.8 index) and equivocal means 18-22 panbio units (1.8-2.2 index). Early detection of elevated IgG levels in secondary infection can be possible from three days following infection, but high values are achieved when samples are obtained between 6 to 15 days after the onset of illness. Hence retesting of samples can be recommended between 4 to 7 days after the first specimen in cases of persistent infection. The limitations are lack of specificity, cross

reaction with other flaviviruses and not suitable for species identification. (Panbio diagnostics).

IgA ELISA:

Dengue specific IgA has become target of interest in Dengue diagnosis. It appears earlier than IgM and IgG and also decreases rapidly. It is a better indicator of recent infection in serum and saliva (non- invasive technique) with sensitivity of 94.4% and specificity of 74.7% in serum, slightly lower in saliva[74].

RAPID DIAGNOSTIC TEST (RDT NS1, IgM and IgG):

This is only a screening test. Example, Dengue day 1 test, is a rapid solid phase immune-chromatographic test for qualitative detection of Dengue NS1 Ag and differential detection of IgM and IgG antibodies.

PRINCIPLE: (ANTIGEN-ANTIBODY REACTION):

Dengue day 1 test kit for NS1 Ag has two lines, control and test lines. Test line is coated with anti-Dengue NS1 antigen. When a sample is added , Dengue NS1 Ag if present in the sample will bind to anti-Dengue NS1 gold colloid conjugate making antigen –antibody complex. This complex migrates along the membrane to the test region and forms the visible pink line (antibody-antigen-antibody gold conjugate complex). The sensitivity is 100% and specificity is 99.94%.

Dengue IgM/IgG test device contains three lines, control line (C), IgM test line (M), and IgG test line (G). Test line of IgG and IgM is coated with anti-human IgG/IgM

monoclonal antibodies. When the sample is added to the well, IgG, IgM antibodies in the sample react with antihuman IgG or IgM antibodies coated on the membrane respectively. Colloidal gold with Dengue 1-4 antigen is captured by bound anti-Dengue IgG or IgM on respective test bands located in the test window causing a pale to dark red band. The sensitivity is 100% and specificity is 99.88%[75,76].

Other rapid tests are Inbios Dengue NS1 (sensitivity 86%, specificity 100%), Biorad NS1Ag strip (sensitivity 72.8%, specificity 100%), Panbio NS1 Ag strip (sensitivity 71.9%, specificity 95%) and SD Dengue dio (sensitivity 70.6%, specificity 100%)[77].

HAEMATOLOGICAL TESTS:

Platelet and haematocrit (HCT) are measured during acute phases of infection. Platelet count < 100000 per μl is observed in DHF in the period between days 3 to 8 following the onset of illness. HCT>20% is indicative of hypovolemia due to vascular permeability and vascular leakage.

FUTURE TEST DEVELOPMENTS:

MICROSPHERE – BASED IMMUNOASSAYS (MIAS):

It is based on covalent bonding of antigen or antibody to microspheres or beads. Detection method include laser to elicit fluorescence of varying wavelengths. It is faster than MAC-ELISA. The advantages of this test is its potential for multiplexing serological tests designed to identify antibody response to several viruses[67].

MASS SPECTROMETRY:

It provides rapid discrimination of biological components in biological mixtures. It produces a specific finger print or molecular profile of virus. The software system built into the instrument identifies and quantifies the pathogen in a given sample. Additionally it recognize a previously unidentified organism, so it is useful in determining not only Dengue serotypes but also genotypes during an outbreak. Samples are processed for DNA extraction, PCR amplification, mass spectrometry and computer analysis[67].

MICROARRAY:

It is possible to screen a sample for many different nucleic acid fragments corresponding to different viruses in parallel. Genetic material must be amplified before hybridization to the microarray, and amplification strategy can target conserved sequence as well as random based ones. Short oligonucleotides attached on microarray slide gives a relatively exact sequence identification. Laser based scanner is commonly used as a reader to detect amplified fragments labelled with fluorescent dye. It is used to test, at the same time, Dengue and other arboviruses responsible for Dengue like symptoms[67].

TREATMENT OF DENGUE INFECTION:

The management of Dengue needs a stepwise approach as follows[67]

- Step I- overall assessment- detailed history, physical examination, investigations including CBC and haematocrit for baseline assessment.

- Additional tests include LFT, RBS, urea and creatinine, serum electrolytes, bicarbonate/lactate, cardiac enzymes, ECG and urine specific gravity

- Step II- lab diagnosis of Dengue, assessment of disease phase(febrile, critical/recovery) and severity by history, physical examination ,CBC, haematocrit, hydration ,haemodynamic status and warning signs present/not based on these parameters whether patient need admission /not
- Step III- management- disease notification, management decisions. Based on the clinical manifestations patients may be categorized into three groups

  Group A- patients can be sent home

  Group B-patient may be referred for in-hospital management

  Group C- require emergency treatment or urgent referral.

TREATMENT OF GROUPS A TO C:

GROUP A:

These patients are able to tolerate oral rehydration and have good urine output without any warning signs. They should be monitored regularly and advised to return to hospital if they develop warning signs. Following protocol can be used.

- Encourage plenty of oral fluids
- For high grade fever give tepid sponging and paracetamol 6^{th} hourly. Do not give NSAIDs as they aggravate gastritis or bleeding and may be associated with Reye's syndrome.
- Advice hospital monitoring if patient develops any of the warning signs.

GROUP B:

This group includes patients with warning signs, associated co morbidities and other social circumstances and they need in-hospital management. The following treatment protocol can be used.

- Obtain haematocrit before fluid therapy. Give isotonic solutions such as 0.9% saline, Ringer's lactate or Hartmann's solution. Infusion should be titrated according to the clinical response.
- Reassess the haematocrit and clinical status and increase or decrease the infusion accordingly.
- Give minimum intravenous fluid volume required to maintain good perfusion and urine output of 0.5 ml/kg/hr.
- Patients with warning signs should be monitored carefully for other organ functions.

GROUP C:

These patients have severe plasma leakage leading to shock, severe haemorrhage and severe organ impairment. They need emergency treatment and urgent referral.

Proper intravenous fluid resuscitation is essential. The crystalloid solution should be isotonic just sufficient to maintain the circulation. Larger volumes should not be infused as the patient may develop pulmonary edema. The primary aim of fluid resuscitation is to improve central and peripheral circulation and also improving end organ perfusion.

TREATMENT OF COMPENSATED SHOCK:

Start IV isotonic crystalloid solution 5 to 10ml/kg/hr for one hour. Then asses the vitals, capillary refill time, haematocrit (HCT). If improved gradually reduce the infusion depending on the hemodynamic status which can be maintained for up to 24 to 48hours. If vital signs are unstable repeat second bolus 10 to 20 ml/kg/hr for one hour. Reduce the infusion rate if there is improvement in patient status. If haematocrit is <40% in children, females and <45% in adult males, it indicates bleeding and needs transfusion.

TREATMENT OF HYPOTENSIVE SHOCK:

Start IV fluid, isotonic crystalloid or colloid 20 ml/kg for 15 minutes. If improved maintain IV crystalloid or colloid 10 ml/kg /hr for one hour. Then maintain according to the haemodynamic status. If there is no improvement review first HCT value. If HCT is high administer second bolus IV colloid 10 to 20 ml/kg over one hour. If HCT is reduced consider bleeding and needs transfusion. If there is no improvement after the second bolus repeat second HCT and manage accordingly. Monitor HCT 6^{th} hourly.

TREATMENT OF HAEMORRHAGIC COMPLICATIONS:

Patients with profound thrombocytopenia advice strict bed rest, protect from trauma and no intramuscular injections. For severe thrombocytopenia prophylactic platelet transfusions should be given.

For severe bleeding give 5 to 10ml/kg of fresh packed red cells or 10 to 20ml/kg of fresh whole blood at an appropriate rate and observe the clinical response. A good

clinical response includes improving haemodynamic status and acid base balance. For further blood loss with reduced HCT transfuse platelet concentrates and FFP.

TREATMENT OF COMPLICATIONS:

FLUID OVERLOAD:

Immediate oxygen therapy. Stop IV fluid. Oral or IV furosemide 0.1 to 0.5 mg/kg/dose once or twice daily can be given. Monitor potassium and correct the ensuing hypokalaemia. If the still remains in shock careful fresh whole blood transfusion should be initiated.

OTHER COMPLICATIONS:

Both hypo and hyperglycaemia can occur. Electrolyte and acid base imbalances are also seen. Should be aware of coinfection and nosocomial infection. All these above mentioned abnormalities should be corrected.

SUPPORTIVE CARE AND ADJUVANT THERAPY FOR SEVERE DENGUE:

Instead of peritoneal dialysis prefer continuous veno-venous haemodialysis (CVVH) for renal replacement therapy. Vasopressors and ionotropic therapies are used to prevent life threatening hypotension, Dengue shock and during induction for intubation. Further treatment of organ impairment should be done.

PREVENTION OF DENGUE INFECTION:

Dengue virus transmission occurs when susceptible hosts, Dengue viruses, and mosquitoes capable of transmission are co-located in space and time.

ENDEMIC AREAS:

Approaches for the prevention of Dengue infection and disease in endemic areas include Aedes mosquito control and vaccine development.

MOSQUITO CONTROL:

Mosquito control is effective but is difficult to sustain. Approaches to mosquito control for prevention of Dengue infection include:

- Reducing breeding sites –by Community-based education to reduce breeding sites that accumulate standing water.
- control of the aquatic stages(larvaland pupal) with temephos(OPC) and Bacillus thuringiensis israelensis (Biological agent) and water vessels can be seeded with copepods /guppy fish(Poecilla reticulate) that feed on mosquito larvae was successful.
- Larva of Toxorhynchites are carnivorus.It is used for the control of Ae.Aegypti and Ae.albopictus larva.
- Larva control Insecticide use- use of insecticide treated curtains-not much effective.
- Endosymbiotic control-release of mosquitoes infected with intracellular endosymbiotic bacterium, Wolbachia can reduce the virustransmission.
- Adulticiding by using Thermal fogs are produced by equipment which mixes an insecticide(pyrethroides) at a relatively low concentration with diesel oil or kerosene as a carrier[78].

VACCINATION:

Generally infection with one serotype provides long term protection with the same serotype and also some short lived protection against other serotypes. Ideal vaccine should provide protective immunity against all serotypes. One of the vaccine is CYD-TDV (DENGVAXIA). It is a formulation of four chimeric yellow fever 17D Dengue vaccine viruses where the premembrane and envelope proteins from each Dengue virus types replaces the same proteins in a yellow fever 17D backbone virus. In one of the trails the vaccine was administered in three doses at months 0, 6, 12. Vaccine efficacy was significantly higher for DENV-3 and DENV-4 (approximately 75%) than DENV-1 (50%) and DENV-2 (35-42%). The follow-up analyses of theses trails shows that the vaccine was associated with increased risk of Dengue infection requiring hospitalization during the third year of trail in children 2 to 5 years of age. Vaccine efficacy was also low[78].

Based on these data it was concluded that the vaccine may yield a public health benefit, though the durability of vaccine impact and level of herd immunity required to affect Dengue transmission are both unknown. According to WHO strategic advisory group of experts the vaccine should be introduced only in geographic settings with seroprevalence> 70% in the target age group. It should not be used if the seroprevalence is <50%.

TRAVELLERS:

The primary preventive approach in travellers include, avoidance of exposure to infected mosquitoes such as remaining in a well screened or air conditioned area during day time. When outside during the day, they should wear protective clothing and use mosquito repellent such as N, N-diethyl-metatoluamide (DEET).

Travellers from non-endemic countries are at low risk of severe Dengue infection in the absence of prior exposure. Exceptions are frequent international travel, military personnel and immigrants from endemic areas returning to their native place.

People with history of Dengue infection need not avoid subsequent travel to endemic regions. Severe Dengue occurs in a small number of secondary infections, so the risk of severe infection is low in travellers[78].

FUTURE DIRECTIONS:

So far there is no data to support the role of corticosteroids, intravenous immunoglobulins, pentoxifylline or activated factor VII. Some direct viral inhibitors are under trail. They are small molecule inhibitors of essential viral enzymes (NS2B-3 protease, NS3 helicase, NS5 methyltransferase, NS5 polymerase) or antibody inhibitors of viral entry/fusion.

Randomized trails of chloroquine, lovastatin, balapiravir (polymerase inhibitor), celgosivir (alpha-glucosidase inhibitor) revealed no significant effects of these drugs or viremia, NS1 antigenemia or fever[78].

MATERIALS AND METHODS

This prospective observational study was conducted in the Department of Microbiology, PSGIMS&R after obtaining the Institutional Human Ethical Committee's approval.

STUDY PERIOD: August 2016 to August 2017

SAMPLE SIZE:

Consecutive serum samples were collected from 150 hospitalized patients and outpatient with history of acute onset of fever less than 10 days duration, who were clinically suspected to have Dengue virus infection in and around Coimbatore.

SAMPLE SIZE JUSTIFICATION:

Formula used: $n= t^2 \times p\ (1-p)/m^2$

Where;

n = required sample size

t = confidence level at 95% (standard value of 1.96)

p = estimated prevalence of *Dengue virus infection in and around Coimbatore*

m = margin of error at 5% (standard value of 0.05)

Estimated prevalence from our hospital statistics (p) = 0.11

n= 1.96 x 1.96 x 0.11 (1- 0.11)/ 0.05 x 0.05

n= 153 (150)

INCLUSION CRITERIA:

As per the WHO classification, the proposed probable diagnosis were,

1. An acute febrile illness with two or more of the following manifestations - headache, retro-orbital pain, arthralgia, rash, hemorrhagic manifestations, leucopenia and a positive IgM, antibody test on serum samples collected five or more days after the onset of fever supports the diagnosis of Dengue.
2. A primary infection was indicated when the IgM to IgG index value ratio was 1.78
3. A secondary infection was indicated when the IgM to IgG ratio was less than 1.78.
4. Taking these criteria into account, patients have been categorized as suffering from primary/secondary infections

EXCLUSION CRITERIA:

1. Fever more than 10 days.

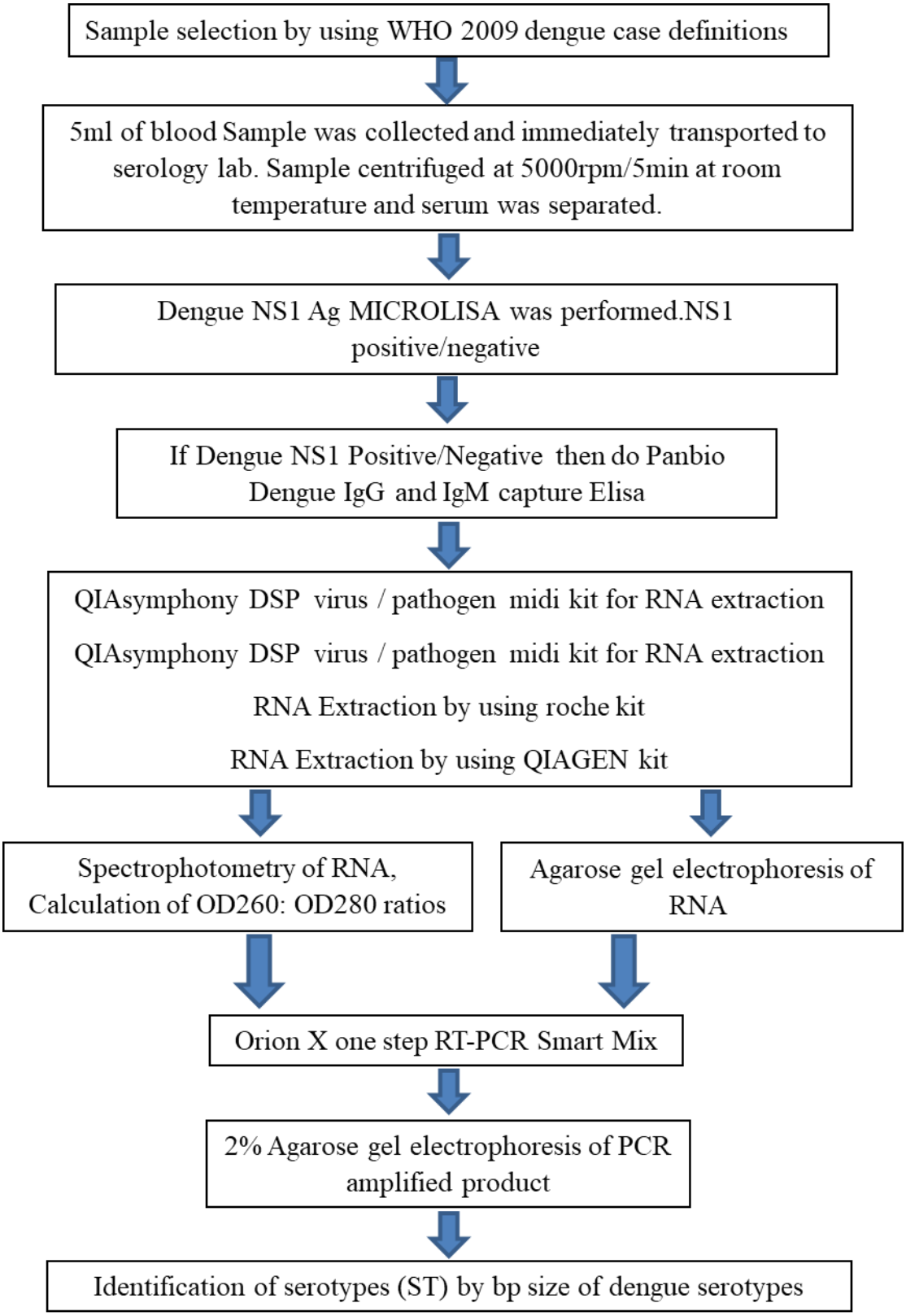
ETHICAL CLEARANCE:

This prospective study was approved by the Ethical Committee of PSG IMS&R.

STATISTICAL ANALYSIS:

Statistical analysis was performed using SPSS software (Statistical Product and Services Solutions, version 17, SPSS Inc, Chicago, Il, USA) to analysis data.

Figure 5: WORK FLOW CHART



SAMPLE COLLECTION:

Blood samples were collected aseptically and serum was separated by centrifugation at 5000rpm/5min in room temperature. Heat inactivated serum, Hemolysis, Icteric and Hyperlipemic process were not tried to avoid test erroneous results.

Sodium azides as preservatives were not used because it inactivates HRPO enzyme**.**

DENGUE NS1 Ag MICROLISA:

It was an in-vitro qualitative detection of NS1 Ag from patients' serum/ plasma. It detects DENV1-4 serotypes.

PRINCIPLE:

It was based on direct sandwich ELISA. Polystyrene surface of micro wells coated with anti-Dengue NS1 antibodies with high reactivity for Dengue NS1 Ag. Samples were added to the wells followed by addition of Enzyme conjugate [monoclonal anti- Dengue NS1 antibodies linked to horseradish peroxidase (HRPO)]. NS1 was trapped/sandwiched between the antibody and antibody HRPO conjugate. Excess unbound conjugate was washed off with wash buffer. The amount of bound peroxidase was directly proportional to the concentration of Dengue NS1 Ag in the sample. Then add colourless TMB (Tetra methyl benzidine) and Chromogen, a blue colour developed by the cleavage of TMB with

HRPO enzymes. The Enzyme-substrate reaction was limited by using Stop solution (1N H2SO4) and yellow colour develops which was read at 450nm spectrophotometrically.

MATERIALS PROVIDED IN THE KIT:

1. Anti NS1 antibody coated micro wells
2. HRPO Conjugated anti NS1 monoclonal antibodies
3. Diluent buffer containing protein stabilizers& antimicrobial agents as preservative
4. Enzyme conjugate concentrate (50X)
5. Wash buffer concentrate (25X)-concentrated phosphate buffer with surfactant.
6. TMB substrate
7. TMB diluent.
8. Positive control-Recombinant Dengue NS1 antigen.
9. Negative control-Normal human serum negative for Dengue NS1.
10. Calibrator- Recombinant Dengue NS1 antigen.
11. Stop solution.
12. Plate sealers-Adhesive sheets to cover micro wells.

MATERIALS NOT PROVIDED IN THE KIT:

1. Micropipettes with disposable micro tips.
2. Deionized water.
3. Micro plate washing system.
4. Micro plate reader with 450nm filter.

5. Timer
6. Graduated cylinder
7. Flask test tubes &plastic tubes or vials for dilutions
8. Incubator 37˚C
9. Disposable gloves
10. Sodium hypochlorite solution

PRE PROCEDURE PREPARATION:

1. Working Wash Buffer: 1 ml of concentrated wash buffer＋24 ml of distilled water (1:25)
2. Working Conjugate: 1 ml of conjugate concentrate＋49 ml of distilled water (1:50).
3. Working Substrate Solution: 1ml of TMB＋1ml of TMB diluent (1:1).

PROCEDURE:

1. Required number of sample wells was taken along with 3 micro wells for 1 positive control, 1 negative control and 1 for calibrator.
2. The positive control, negative control, calibrator and serum samples were diluted with sample diluent by adding 50µl of controls/samples with 50µl sample diluent in each well.
3. Added 100µl of working conjugate solution in each well and mixed well
4. Incubated at 37˚C for 90 min.
5. Then wells were washed with working wash buffer for 6 times.
6. Add 150µl of working substrate solution in each well.

7. Incubated at 25˚C for 30 min in dark.
8. Add 100µl stop solution.
9. Read absorbance at 450nm with a reference filter of 600-650 nm within 30 min in ELISA Reader

CALCULATION OF RESULTS:

VALIDITY OF THE TEST: Negative O.D must be <0.3, Positive O.D >1.0, Mean calibrator O.D>/=0.35,Cut- off value >/=1.5 x NC O.D and Ratio of PC O.D/Cut off must be >1.1.

a) Calculation of the triplicates of Calibrator 'mean and multiply' by the calibration factor. Cut off value= mean O.D of calibrator x calibration factor. Calibration factor was batch specific. Value was 0.7
b) An index value can be calculated by dividing the sample absorbance by the Cut-off value (calculated in step A). Index value= Sample O.D/Cut-off value.
c) NS1 Ag units can be calculated by multiplying the index valuex10 (as in step B)

Table 4: Interpretation of results of Dengue NSI

INDEX VALUE	**NS1 Ag UNITS**	**RESULTS**
＜0.9	＜ 9	Negative
0.9-1.1	9-11	Equivocal
＞ 1.1	＞ 11	Positive

Sensitivity: 99.5% Specificity: 100%

PANBIO DENGUE I_gG CAPTURE ELISA:

PRINCIPLE:

Serum antibodies of the IgG class, when present, combines with anti- human IgG antibodies coated on micro well test strips. A concentrated recombinant Dengue 1-4 Antigens was diluted to correct working volume with antigen diluent. An equal volume of HRPO - Conjugated monoclonal antibody (MAb) was added to the diluted antigen, allowing to form antigen- MAb complexes residual serum was removed by washing. This complex binds with serum Dengue specific IgG antibodies. After incubation the micro wells were washed and a colourless substrate (TMB Chromogen) was added. The substrate was hydrolyzed by HRPO and the chromogen turns blue. Then stop solution was added, the TMB turns yellow. Colour development was indicative of presence of anti-Dengue IgG antibodies in patient's serum.

MATERIALS PROVIDED IN THE KIT:

1. Anti-human IgG-coated Micro wells
2. Dengue 1-4 Recombinant Antigens
3. Wash buffer-Phosphate buffer with Tween 20 and preservatives (0.1%proclin)
4. Sample Diluent-Tris buffer saline
5. Antigen diluent-Phosphate buffer
6. HRPO with MAb Tracer
7. TMB Chromogen
8. Dengue IgG Reactive control-human serum

9. Dengue IgG Capture Calibrator –human serum
10. Dengue IgG capture Negative control-human serum
11. Stop solution- 1M Phosphoric acid

MATERIALS REQUIRED:

1. Accurate adjustable micropipettes with disposable micro tips
2. Deionized water
3. Micro plate washing system.
4. Micro plate reader with 450nm filter.
5. Timer
6. Graduated cylinder
7. Flask test tubes & plastic tubes or vials for dilutions
8. Incubator 37˚C
9. Disposable gloves
10. Sodium hypochlorite solution

PRE TEST PREPARATION:

WORKING WASH BUFFER PREPARATION:

Concentrated wash buffer 25 ml＋Distilled water 475ml (1:20 dilution**)**

PROCEDURE

1) Required number of sample wells were taken along with 3 wells for 1 positive control, 1 Negative control, 1 micro well for calibrator.

2) The PC, NC, Calibrator and Serum sample were diluted with sample diluent, by adding10µl of each with 1ml sample diluent in clean glass test tubes.
3) Add 100 µl of this 1: 100 dilution samples and controls into respective wells. If serum with IgG antibodies were attached to anti-human IgG antibodies coated in assay plate
4) The plate was covered and incubated at 37˚C for one hour. After incubation, these wells were washed for 6 times by using wash buffer
5) Dilute the Antigen 1/250 by using antigen diluents (10 µl of Ag with 2.5 ml of Ag diluent).
6) Removed required amount of Ag and mix with an equal volume of MAb Tracer in separate vials. Incubate at 20-25˚C for one hour
7) Transfer 100µl of Ag-MAb per well to assay plate. Cover plate and incubate 1hour at 37˚C.Then wash the assay plate for 6 times
8) Add 100 µl TMB in each well and incubate at 20-25˚C (dark) for10 minutes
9) Then add 100 µl stop solution
10) Read at 450 nm (Reference 600-650 nm).

CALCULATION OF RESULTS:

VALIDITY OF THE TEST: Negative control O.D must be <0.400, Positive control/cut off ratio 1.1 – 7.0, Cut- off value >/=1.5 x NC O.D.

a) Calculation of the triplicates of Calibrator mean and multiply by the calibration factor. Cut off value= mean O.D of calibrator x calibration factor. Calibration factor was batch specific. Value was 0.77
b) An index value can be calculated by dividing the sample absorbance by the Cut-off value (calculated in step A). Index value＝ Sample O.D/Cut-off value.
c) Panbio units can be calculated by multiplying the index valueX10 (calculated in step B)

Table 5: Interpretation of results of Dengue IgG

INDEX VALUE	**PANBIO UNITS**	R**ESULTS**
＜1.8	＜ 18	Negative
1.8-2.2	18-22	Equivocal
＞ 2.2	＞ 22	Positive

PANBIO DENGUE IgM CAPTURE ELISA:

It was for the qualitative detection of IgM antibodies to Dengue antigen in serum. It could be detected 3to5 days after the onset fever and persists for 30 to90 days. Sometimes detectable levels may be present upto 8 months from the onset of fever.

PRINCIPLE:

Serum antibodies of the IgM class, when present, combine with anti- human IgM antibodies coated on micro well test strips. A concentrated recombinant Dengue 1-4 Antigens was diluted to correct working volume with antigen diluent. An equal volume of HRPO - Conjugated monoclonal antibody (MAb) was added to the diluted antigen, allowing to form antigen- MAb complexes. Residual serum was removed by washing. This complex binds with serum Dengue specific IgM antibodies. After incubation the micro wells washed and a colourless substrate (TMB Chromogen) was added. The substrate was hydrolyzed by HRPO and the chromogen turns blue. Then stop solution addition. The TMB turns yellow. Colour development was indicative of presence of anti-Dengue IgM antibodies in patient's serum.

MATERIALS PROVIDED IN THE KIT:

1. Anti-human IgM-coated Micro wells
2. Dengue 1-4 Recombinant Antigens
3. Wash buffer-Phosphate buffer(pH7.2-7.4) with Tween 20 and preservatives(0.1%proclin)
4. Sample Diluent-Tris buffer saline

5. Antigen diluent-Phosphate buffer
6. HRPO with MAb Tracer
7. TMB Chromogen
8. Dengue IgM Reactive control-human serum
9. Dengue IgM Capture Calibrator –human serum
10. Dengue IgM capture Negative control-human serum
11. Stop solution- 1M Phosphoric acid

MATERIALS REQUIRED:

1. Accurate adjustable micropipettes with disposable micro tips
2. Deionized water
3. Micro plate washing system.
4. Micro plate reader with 450nm filter
5. Timer
6. Graduated cylinder
7. Flask test tubes &plastic tubes or vials for dilutions
8. Incubator 37˚C
9. Disposable gloves
10. Sodium hypochlorite solution

PRE TEST PREPARATION:

WORKING WASH BUFFER PREPARATION:

Concentrated wash buffer 25 ml ＋ Distilled water 475m l (1:20 dilution**)**

PROCEDURE:

1) Required number of sample wells was taken along with 3 wells for 1 positive control, 1 Negative control, 1 micro well for calibrator.
2) The PC, NC, Calibrator and Serum sample were diluted with sample diluent, by adding 10µl of each with 1ml sample diluent in clean glass test tubes.
3) Add 100µl of this 1: 100 dilution controls and samples into respective wells. Serum with IgM antibodies were attached to anti-human IgM antibodies coated in assay plate.
4) The plate was covered and incubated at 37˚C for one hour. After incubation, these wells were washed for 6 times by using wash buffer.
5) Dilute the Antigen 1/250 by using antigen diluents (10µl of Ag with 2.5ml of Ag diluent)
6) Remove required amount of Ag and mix with an equal volume of MAb Tracer in separate vials .Incubate at 20-25˚C for one hour
7) Transfer 100µl of Ag-MAb each wells to assay plate. Cover plate and incubate 37˚C. Then wash the assay plate for 6 times
8) Add 100µl TMB in each well and incubate at 20-25˚C for10 minutes in dark

9) Then add 100µl stop solution

10) Read at 450nm (Reference 600-650nm)

CALCULATION OF RESULTS:

Validity of the test: Negative O.D must be <0.400, Positive cut off ratio 1.1 – 8.0, Cut-off value >/=1.5 x NC O.D

a) Calculation of the triplicates of Calibrator 'mean and multiply' by the calibration factor. Cut off value= mean O.D of calibrator x calibration factor. Calibration factor was batch specific. Value was 0.96

b) An index value can be calculated by dividing the sample absorbance by the Cut-off value (calculated in step A). Index value＝ Sample O.D/Cut-off value

c) Panbio

Units can be calculated by multiplying the index valuex10 (calculated in step B)

Table 6: Interpretation of results Dengu IgM

INDEX VALUE	**PANBIO UNITS**	R**ESULTS**
＜0.9	＜ 9	Negative
0.9-1.1	9-11	Equivocal
＞ 1.1	＞11	Positive

RNA EXTRACTION METHODS:

To find out serotypes prevalence in this region various RNA extraction methods were tried to find out which was best method to isolate RNA, out of 150 continuous study samples 22 of NS1 Ag units was showed high value. These 22 samples were processed for molecular study. Finally I found out QIA symphony DSP virus / pathogen midi kit for RNA extraction was best method to isolate RNA from sample.

QIASYMPHONY DSP VIRUS / PATHOGEN MIDI KIT

PRINCIPLE:

It comprises four steps including lyse, Bind, Wash & Elute. This technology combines the speed and efficacy silica-based nucleic acid purification with the convenient handling of magnetic particles. It was a safe and reproducible handling of potentially infectious samples by M/s QIAGEN GmbH, Germany.

AIM:

- Automated isolation and purification of nucleic acids from biological specimens utilizing magnetic particle technology
- Purification- high quality nucleic acid free of proteins, nucleases, and other impurities.

REAGENTS & MATERIALS PROVIDED

RC-Reagent Cartridge has guanidine salts which form highly reactive compounds when combined with bleach.

1- ER-Enzyme Rack
2- PL-Piercing Lid
3- AVE-Buffer AVE
4- AVE- Buffer AVE
5- CARRIER-Carrier RNA
6- Proteinase K
7- RSS-Reuse Seal Set

MATERIALS REQUIRED:

1- Sample prep Cartridge,8-well
2- 8-Rod covers
3- Filter-Tips,200µl and 1500µl
4- Sample tubes
5- Vortex

For pathogen complex protocols

Buffer ATL, internal controls, Sample tubes, 14ml 17x100 polystyrene, round – bottom tubes from BD, Sample tubes 2 ml, with screw caps / without screw caps.

Figure 6: QIAsymphony RNA extraction method

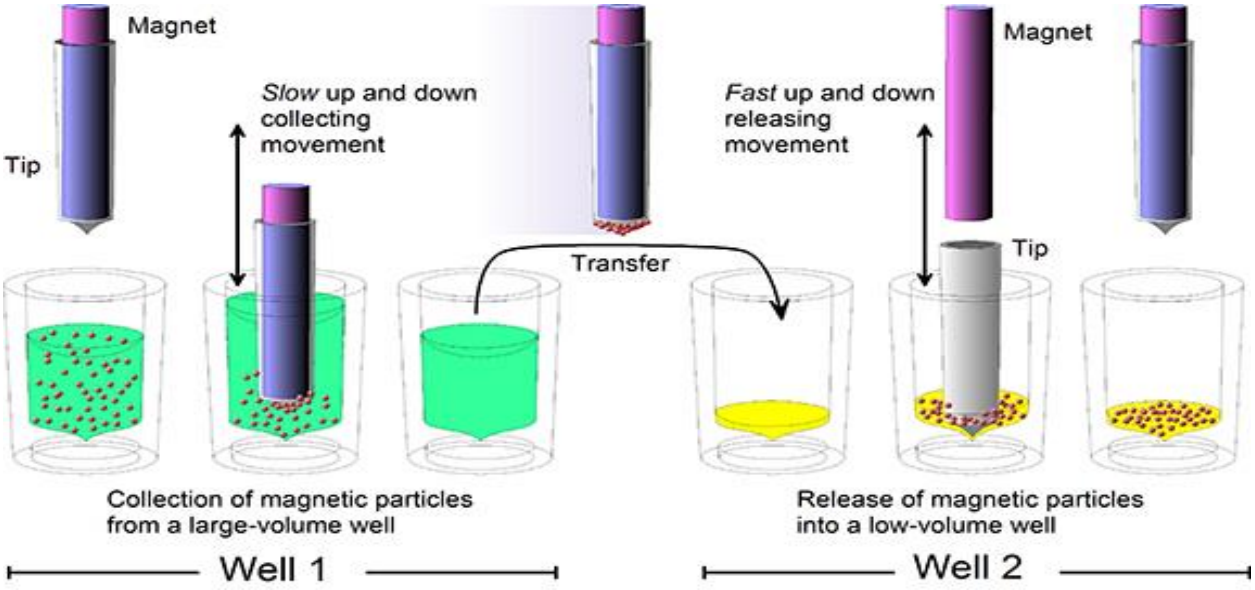
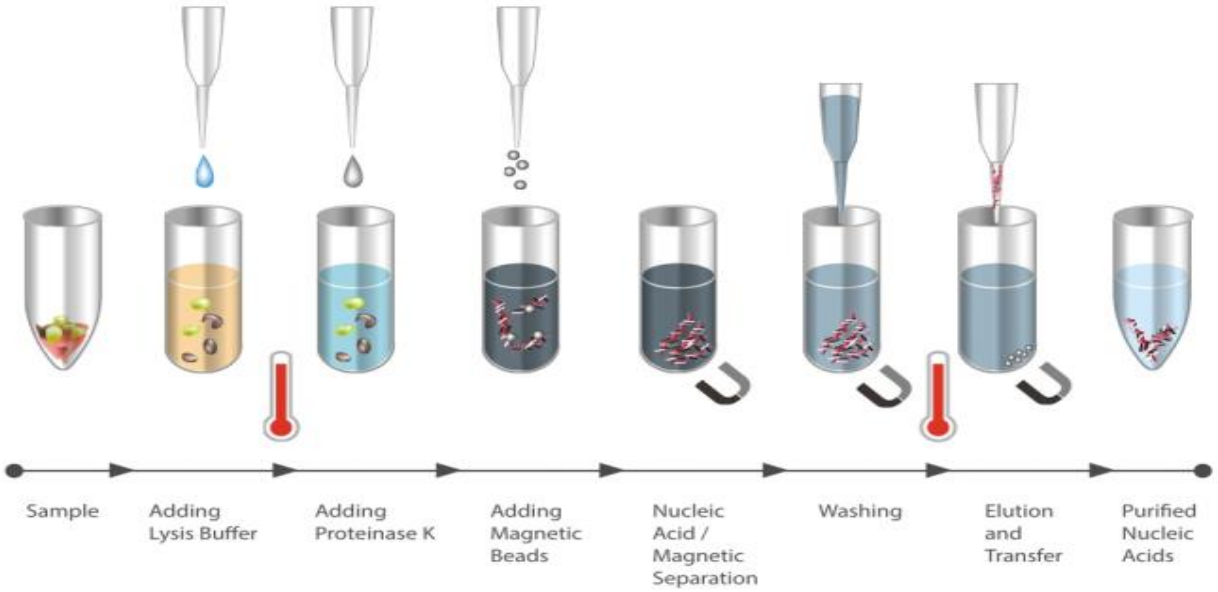


Figure 7: Dengue RNA extraction and purification



THINGS DONE BEFORE STARTING:

Prepared all required mixtures, including mixtures containing carrier RNA (CARRIER) and internal controls (optional) just before starting.

Make sure PL placed on RC and removal of lid of magnetic particles and open the enzyme tubes

Barcodes if used for samples to be pasted and face in correct orientation.

PROCEDURE:

The QIAsymphony SP makes automated sample preparation which was easy and convenient.

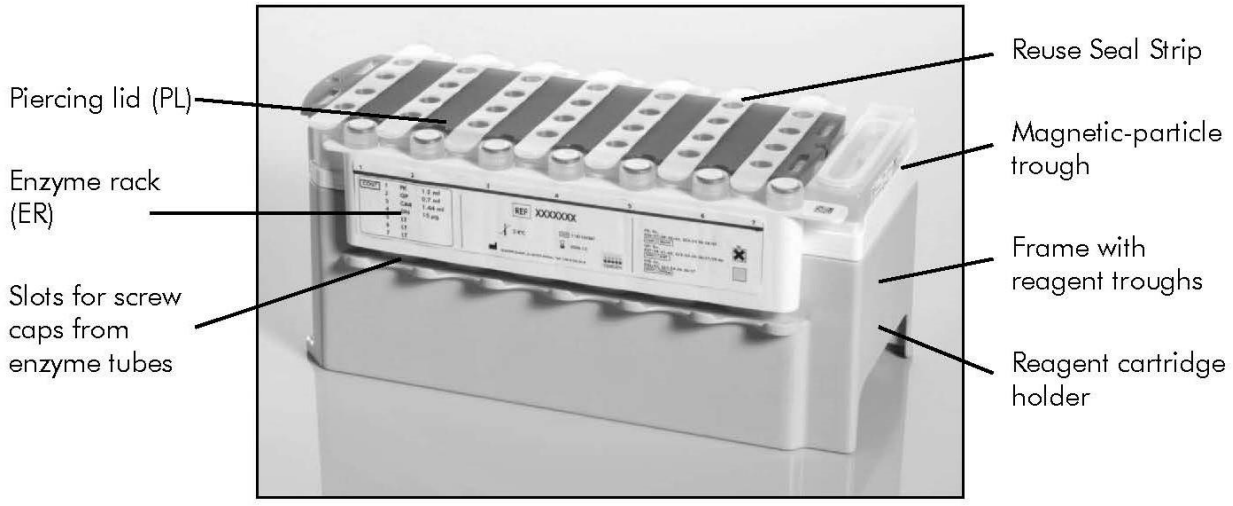
Kits were suitable for use with a wide range of sample types, including plasma, serum, CSF, and respiratory and urogenital samples. Samples, reagents and consumables, and elutes were separated in different drawers. Preparation of IC (Internal Controls) which contains carrier RNA 3µl, molecular biological grade water 14µl and buffer AVE 10µl per sample.

Finally calculated the number of samples of the day run with addition of dead volume of three samples. Then opened the sample drawer and placed the IC rack in the right end of A position. Placed the tubes containing the carrier RNA-Buffer AVE mixture (including optional internal control) into slot A of the sample drawer and sample carrier in the sample slot. Started the RUN button to start the purification process. Finally retrieved elution rack containing the purified nucleic acid from the eluate. Then

store at 2~8˚C for short term storage upto 24hrs. For long term storage, it will be -20˚C. Then close the instrument drawer and switch off machine.

Simply load samples (1.5ml), reagents provided in special cartridges, and pre-racked consumables in the appropriate drawer before run. Start the protocol and remove purified nucleic acids (60µL) from the "Eluate" drawer after processing.

Figure 8: **QIAsymphony reagent troughs**



LOW YIELD OF NUCLEIC ACID DUE TO,

a) Magnetic particles were not completely re-suspended
b) Frozen samples were not mixed properly after thawing
c) Carrier RNA (CARRIER) not added
d) Degraded nucleic acids
e) Incomplete sample lysis
f) Clogging of pipet tip due to insoluble material

From this test, only 14 samples had the ratio of OD_{260}:OD_{280} more than 1.8 and 2.0 respectively. Hence all these 14 samples subjected to complementary DNA conversion and amplification.

HIGH PURE RNA ISOLATION ROCHE KIT:

PRINCIPLE:

Serum /plasma lysed by binding buffer. Nucleic acid was bound to glass fibres pre-packed in the high pure filter tube. Bound nucleic acid were washed with

KIT CONTENT:

1- Binding buffer-guanidine-HCL, Tris –HCL, Triton X

2- Poly (A)-Carrier RNA for binding of RNA

3- Inhibitor removal buffer-add with absolute ethanol, guanidine –HCL

4- Tris-HCL, p H-7.5

5- Wash Buffer-add absolute ethanol, NaCL, Tris-HCL, p H-7.5

6- Elution buffer-add PCR grade water

7- High Pure Filter tubes- up to 700µl

8- Absolute ethanol

9- Micro centrifuge tubes 1.5 ml, sterile(Nuclease free)

10- Collection Tubes-2ml

11- Sample material-200 to 600 µl of serum, plasma, urine, or cell culture supernatant

PROCEDURE:

Add 200µl of serum in 400µl PBS (phosphate buffer solution) with Poly (A). Vortex it for 1 minute then to transfer the sample to a High Pure Filter Tube which was inserted into one collection tube. Pipet the entire sample into the upper reservoir of the Filter Tube (max 700µl). Insert the entire High Pure Filter Tube assembly into a Standard table-top centrifuge. Centrifuge the tube assembly at 8000rpm/1min. After centrifugation, remove the Filter Tube from the Collection tube. Discard the flow through, and again combine the Filter tube and the used Collection tube. After re-inserting the Filter Tube, then add 500µl of inhibitor removal buffer to the upper reservoir. Centrifuge the tube assembly at 8000rpm/1min**.**

Pipet the 450µl Wash Buffer to the upper reservoir of the filter Tube assembly and centrifuge 8,000 rpm/1min. Discard the flow through and combine the Filter Tube with the used Collection Tube. Again 450µl Wash Buffer to the upper reservoir of the filter Tube assembly and centrifuge 8,000 rpm/1min. Discard the flow through and combine the Filter Tube with the sterile Collection Tube. To elute the RNA, Add 50µl Elution Buffer to the upper reservoir of the Filter Tube. Centrifuge the tube assembly at 13,000rpm /1m. Filter Tube assembly and centrifuged for another 1 min at 13,000rpm to remove any residual Wash Buffer. The micro centrifuge tube contains the eluted, purified RNA, which can be used directly in RT-PCR or stored at ‑80°C for later analysis.

RNA EXTRACTION BY USING QIAGEN KIT:

EXTRACTION STEPS:

Added 560µl of prepared buffer AVL to a clean 1.5 ml tube with 5.6µl of carrier RNA to this addition of 140µl of sample to the same tube which was incubated at room temperature for 10 min. In this 560µl of ethanol was added and mixed by vortex. From this transferred 630 µl of Lysate into upper reservoir of spin column. Closed it and centrifuged at 8000rpm/ 1min.transfer spin column tube to a new 2ml collection tube. Add 500µl of AW1 and centrifuge at 8000rpm/1min.Then spin column tube was transferred to a new 2ml collection tube. Then added 500µl of AW2 and centrifuge at 14,000rpm/4min and transfering spin column tube to a new 1.5 ml tube for elution. Added 60µl of elution buffer and kept it for 5min at room temperature, centrifuge it 10,000rpm /1min. Finally eluted RNA was collected in 1.5 ml tube and discard the spin column tube. This eluted Genomic RNA was directly used for RNA agarose formaldehyde gel run and remaining RNA elute was stored at -20˚C for further conversion of cDNA.

SPECTROPHOTOMETRY OF RNA:

It was rapid, simple and non-destructive absorption Spectroscopy has been the method of choice to measure the amount of RNA and DNA in the concentrated pure solution by taking reading at 260nm. For quantitating the amount of RNA, readings were taken at Wave Length (WL) of 260nm and 280nm. The concentration of nucleic acid (NA) in the sample was calculated by readings taken at 260nm. An OD of 1 corresponds

to ~50µg/ml for ds DNA, 40µg/ml for RNA. The Ratio between readings at 260nm and 280nm (OD_{260}:OD_{280}) provides the purity of the NA. The values of 1.8 and 2.0 respectively. If there was a contamination of protein or phenol, OD_{260}:OD_{280} will be less than the above value.

By using this method, quantification of RNA done for 150 samples progressively special inhibitor removal buffer to get rid of RT-PCR inhibitory contaminants. It allows even the application of heparinized sample material with >100u/ml heparin. Washing of bound nucleic, purification from salts, protein and other cellular impurities. Purified nucleic acids were recovered using the elution

MOBS BUFFER GEL ELECTROPHORESESIS USING FORMALDEHYDE:

By using this method, quality of the isolated RNA will be checked. Pathogenic RNA Vs Micro RNA in the samples were identified. Micro RNA show less than 100 bp size while pathogenic RNA exhibits more than 100bp size.

ORION X ONE STEP RT-PCR SMART MIX (2X):

RT-PCR was done for 14 isolates which had OD ratio of OD260:OD280 more than 1.8 and 2.0 respectively. OrionX One Step RT-PCR smart mix was a product for easy reverse transcription and PCR at “One Step” with optimal recombinant of Reverse Transcriptase and HotStart h-Taq DNA polymerase mixture. This kit has dual advantage of high efficacy for cDNA synthesis and high specificity of hot start polymerase to setup

of optimal PCR conditions easily and conveniently from M/s Origin Diagnostics, Thiruvananthapuram, Kerala. The kit can be stored at -20°C.

PRINCIPLE:

Extracted Dengue viral RNA from the serum samples was converted to cDNA by using reverse primer D2 and reverse transcriptase enzymes. Within a single tube, cDNA was amplified by using universal Dengue viral D1&D2 primers. Identification of the four serotypes was achieved by nested amplification of a primary product generated with D1&D2.

The expected size of the RT-PCR products was 511 bp by using universal Dengue viral primers D1&D2. Expected size of each amplicons for the each Dengue serotypes were TS1 – 482 bp, TS2 – 119 bp, TS3 - 290 bp and TS4 – 392 bp. The primers were diluted 1:10 with molecular grade water before use. The remaining volume was adjusted with PCR grade water.

Table 7: Dengue Primers used in this study were:

Gene	Primer sequence (5'-3')	Amplicon size (bp)	Reference
D1	5'-TCAATATGCTGAAACGCGCGAGAAACCG-3'	511	
D2	5'-TTGCACCAACAGTCAATGTCTTCAGGTTC-3'	511	
TS1	5'-CGTCTCAGTGATCCGGGGG-3'	482 (D1 & TS1)	
TS2	5'-CGCCACAAGGGCCATGAACAG-3'	119 (D1 & TS2)	
TS3	5'-TAACATCATCATGAGACAGAGC-3'	290 (D1 & TS3)	
TS4	5'-CTCTGTTGTCTTAAACAAGAGA-3'	392 (D1 & TS4)	

FEATURES OF ONE STEP KIT:

RTase	M-MLV RTase (RNase H^-)
Temperature of cDNA synthesis	42-55 ˚C
Synthesis length	<1 kb
Synthesis primer	Gene specific primer
Expiration date	1 year

Other materials required:

- Cell free samples (such as Plasma or serum samples)
- Vortex mixer
- Cold (4˚C)Micro centrifuge
- 0.5 ml Eppendorf tubes
- Micropipettes (100-1000µL, 10-100µL, 1-10µL)
- Sterilized Microtips (100-1000µL, 10-100µL, 1-10µL)
- Molecular grade water
- The eluted cDNA amplified product was stored in 0.5ml Eppendorf tubes at -20°C.

PROCEDURE:

The Single step Nested RT- PCR was performed by using cDNA production in the conventional method using Applied Bio systems Step One Real-time PCR system immediately after the extraction purified viral RNA.

After preparation of PCR mixture with total volume of 30µl as mentioned below the table, the PCR cycle was initiated with initial holding temperature 50˚C for 30 minutes in single cycle for conversion of cDNA followed by denaturation was done at 95°C for 15 minutes in a single cycle followed by 95°C for 20 seconds followed by annealing temperature 55°C for 45 seconds in 40 cycles and followed by extension at 72°C for 60 seconds and a final elongation at 72°C for 5 minutes. Identification of the four serotypes was achieved by Single step nested amplification of a primary product generated with D1&D2.Below table shows the recommended PCR mixture and cycling:

Table 8: Orion X -one step PCR mixtures and cycle for Dengue Serotyping

PCR mixture (Reaction Vol. 30µL)		Cycle		
Orion X one step RT-PCR Smart Mix (2X)	15 µL	50˚C	30 min	x1
Forward Primer (10pmole/ µL)	1µL	95˚C	15 min	x1
Reverse Primer (10pmole/ µL)	1µL	95˚C	20 Sec	
Template RNA	3µL	AT (55˚C)	40 Sec	x 35-40
TS1 Primer (10pmole/ µL)	1µL	72˚C	1 min	
TS2 Primer (10pmole/ µL)	1µL	72˚C	5 min	x1
TS3 Primer (10pmole/ µL)	1µL			
TS4 Primer (10pmole/ µL)	1µL			
Add D.W to	30µL			

The amplified products were stored at -20°C until they were subjected to gel electrophoresis.

DETECTION OF PCR PRODUCTS BY AGAROSE GEL ELECTROPHORESIS:

The amplified products were visualized by agarose gel electrophoresis. 2% agarose gel was prepared by adding nuclease and protease free agarose powder with 1x Tris Borate EDTA (TBE) buffer. This mixture was heated in the microwave until it formed a clear solution and 0.2µL of ethidium bromide was added to visualise the amplified DNA under UV light. The mixture was allowed to set in an electrophoresis tank with a comb in place.

5µl of a 100 base pair DNA ladder was used as the molecular marker to measure the size of the amplified product and was added in the first well. The rest of the wells had 25µL of the amplified product with loading dye 3µL per sample. Gel electrophoresis was performed by placing the gel in an electrophoresis tank containing 1x TBE buffer at 70 volts for 2 hour.

INTERPRETATION:

Following electrophoresis, images of the gel were captured by Gel Doc. By comparing with the 100 base pair ladder, the size of the amplified product was measured and noted down.

RESULTS

The total number of patients with suspected Dengue in our hospital from August 2016 to August 2017 was 6557. All samples were tested for Dengue IgM and IgG antibodies by using Dengue IgM and IgG capture ELISA and /orNS1 Antigen depending on the duration of fever. NS1 was tested in cases with fever of less than five days duration. With these parameters the total seropositivity was 70.81 % (4643).

Figure 9: Total seropositive Dengue cases recorded during study Period

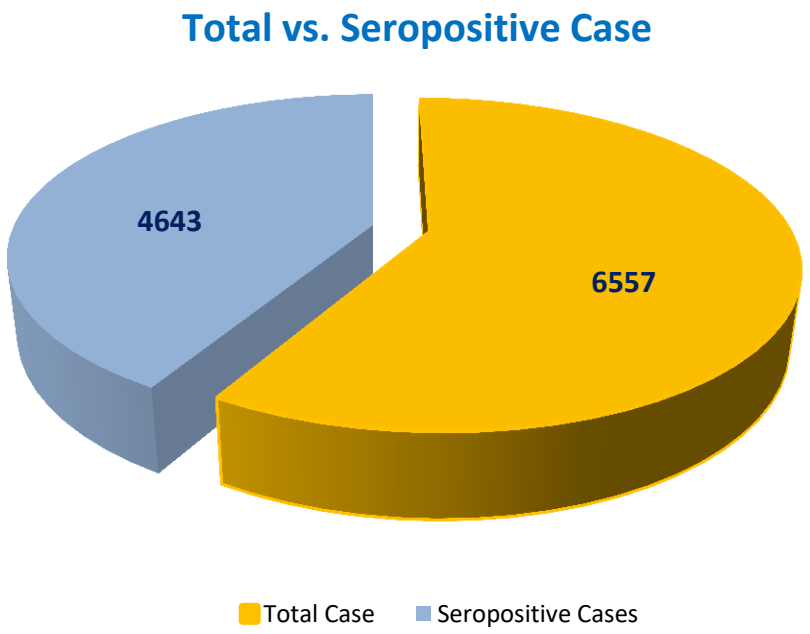
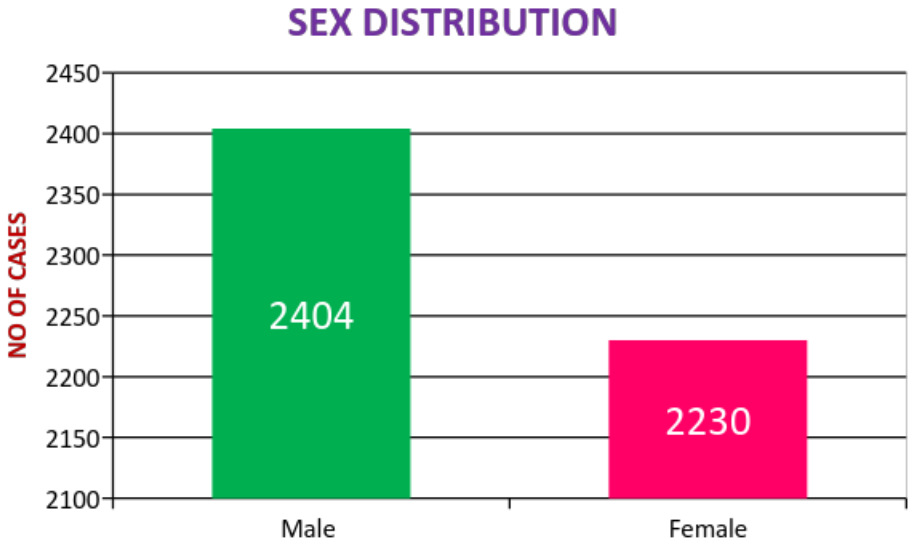
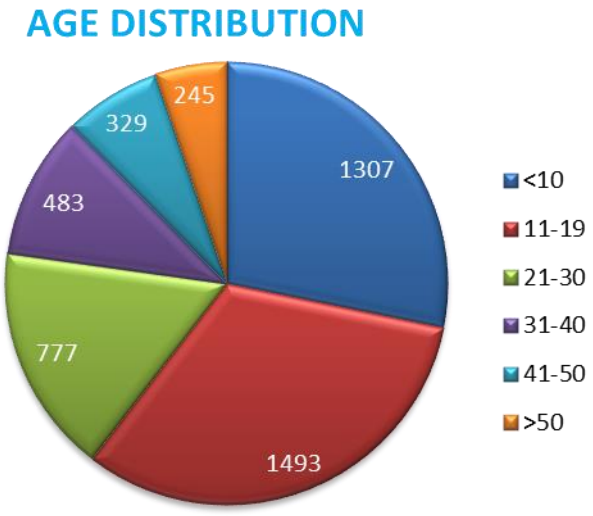


Figure 10: Male to female sex distribution in Dengue



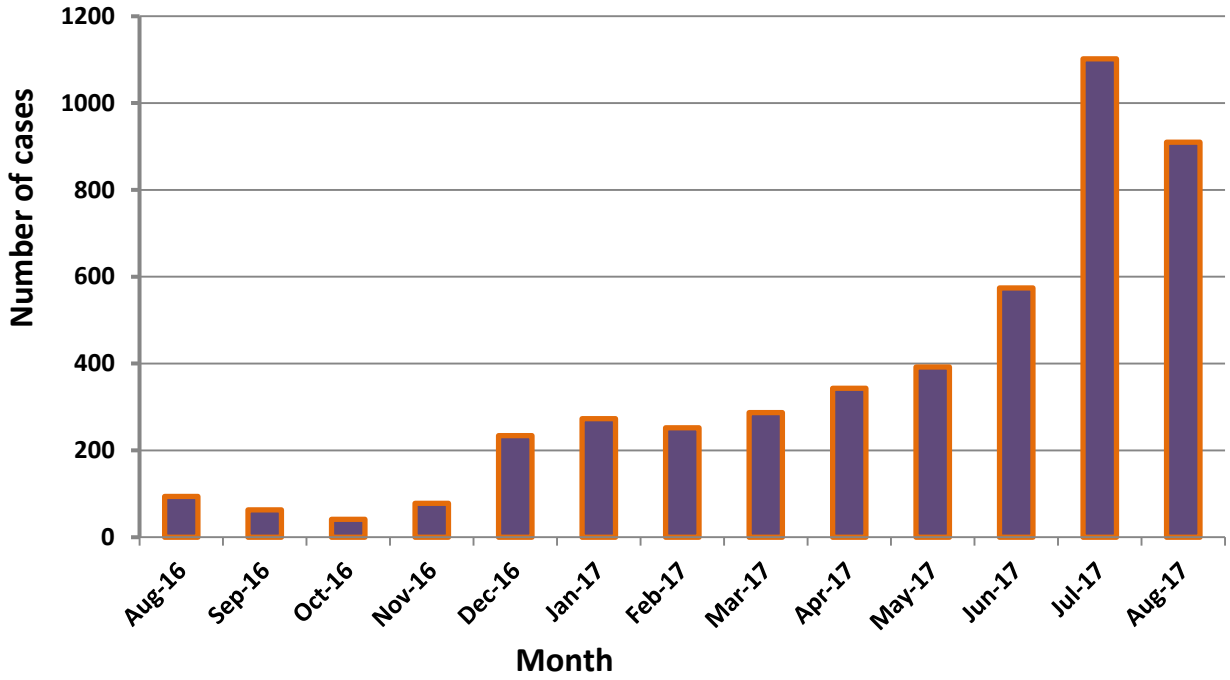
It was observed that no significant difference in male to female sex ratio. Only slight increase in male patients was seen.

Figure 11: Age dependent distribution of Dengue cases



The above data shows that patients in the age group between 11 to 19 years were commonly affected.

Figure 12: Month wise distribution of Dengue seropositive cases



It was observed from the above data that more number of cases was seen during the months of July and August.

RESULTS OF NS1 ANTIGEN MICROLISA:

Consecutive 150 serum samples were collected from patients who had $\leq$ 9 days of fever and subjected to NS1 antigen detection. NS1 antigen detection was done by using Microlisa. Clinical data was collected from NS1 antigen positive patients and parameters like sex distribution, platelet counts, liver enzymes, WBC counts, packed cell volume and duration of fever were assessed.

Table 9: Primary Dengue infection with WHO clinical classification

Serological test	Dengue without Warning signs	Dengue with warning signs	Severe Dengue	Total
NS1 Only	31 (63.26%)	10 (76.92)	3 (75%)	44
NS1+IgM	8 (16.33%)	2(15.38 %)	1 (25%)	11
IgM Only	10 (20.41%)	1 (7.70 %)	-	11
Total	49	13	4	66

Table 9 shows NS1 only positivity was high when compared to other parameters. In Dengue without warning signs NS1 Positivity was 63.26%(31), with warning signs 76.92%(10) and 75%(3) of severe Dengue.

Table 10: Secondary Dengue infection with WHO clinical classification

Serological test	Dengue without Warning signs	Dengue with warning signs	Severe Dengue	Total
NS1+IgM+IgG	6 (17.65%)	4 (25%)	1 (50%)	11
IgM+IgG	27 (79.41%)	12(75 %)	1 (50%)	40
IgG Only	1(2.91%)	-	-	1
NS1+IgG	-	-	-	0
Total	34	16	2	52

Table 10 shows high IgM+IgG positivity in secondary Dengue when compared to other parameters irrespective of the clinical severity. IgM+IgG positivity in Dengue

without warning signs is 79.41 % (27), with warning signs is 75 % (12) and 50 % (1) in severe Dengue.

Table 11: Distribution of platelet counts in primary Dengue

Platelet count	Dengue without warning sign	Dengue with warning sign	Severe Dengue
<150x 10^3	30 (61.22)	10 (83.33)	4 (100%)
150-400 x 10^3	18 (36.73)	1 (8.33)	0
>400 x 10^3	1(2.05)	1 (8.33)	0

Among the study population, 61.22 % (30) cases of Dengue without warning signs, 83.33 % (10) cases of Dengue with warning signs and 100 % (4) of severe Dengue had thrombocytopenia.

Table 12: Distribution of platelet counts in secondary Dengue

Platelet count	Dengue without warning sign	Dengue with warning sign	Severe Dengue
<150x 10^3	30 (88.23)	15 (93.75)	2 (100%)
150-400 x 10^3	3 (8.82)	1 (6.25)	0
>400 x 10^3	1(2.95)	0	0

Among the study population 88.23 % (30) cases of Dengue without warning signs, 93.75 % (15) cases of Dengue with warning signs and 100 % (2) of severe Dengue had thrombocytopenia. Hence thrombocytopenia was seen in majority of Dengue cases, serial monitoring of platelet count is mandatory.

Table 13: Packed cell volume (PCV) in primary Dengue infection

PCV	Dengue without Warning sign	Dengue with warning sign	Severe Dengue	Total
< 35 %	8 (16.33%)	4 (30.77 %)	1 (25 %)	13
35-45 %	33 (67.35 %)	7 (53.85 %)	1 (25 %)	41
>45 %	8 (16.32 %)	2 (15.38 %)	2 (50%)	12
	49	13	4	66

Table 13 shows that 50 % (2) cases of severe Dengue had elevated packed cell volume in primary Dengue.

Table 14: Packed cell volume (PCV) in Secondary Dengue infection

PCV	Dengue without Warning sign	Dengue with warning sign	Severe Dengue	Total
< 35 %	4 (11.76%)	3 (18.75 %)	0	7
35-45 %	25 (73.53 %)	12 (75 %)	0	37
>45 %	5 (14.71 %)	1 (6.25 %)	2 (100%)	8
	34	16	2	52

Table 14 shows that 100 %(2) cases of severe Dengue had elevated packed cell volume in secondary Dengue. Normal PCV is in the range of 40-45 % for men and 35-40 % for women. Hence significantly elevated PCV were seen in severe Dengue, more in cases of secondary Dengue

Table 15: White blood cell (WBC) count in Dengue Primary and secondary infections

WBC count/ µL	Primary			Secondary		
	Dengue without warning signs	Dengue with warning signs	Severe Dengue	Dengue without warning signs	Dengue with warning signs	Severe Dengue
< 4000	16 32.65%)	1 (7.69%)	1 (25%)	13 (38.23%)	0	1 (50%)
>4000 - 11, 000	30 (61.23%)	10 (76.92%)	3 (75%)	20 (58.82%)	15 (93.75%)	1 (50%)
>11000	3 (6.12%)	2 (15.38%)	0	1 (2.94%)	1 (6.25%)	0
	49	13	4	34	16	2

Table 15 shows that there was no significant leukocytosis or leukopenia in primary/ secondary Dengue, irrespective of the clinical severity.

Table 16: Duration of fever in Dengue infection

Fever duration (in days)	Dengue without warning signs		Dengue with warning signs		Severe Dengue	
	Primary	Secondary	Primary	Secondary	Primary	Secondary
≤ 4 days	41 (83.67%)	21 (61.76%)	5 (38.46%)	6 (37.50%)	0	0
> 4 days	8 (16.33%)	13 (38.24%)	8 (61.54%)	10 (62.50%)	4 (100%)	2 (100%)
	49	34	13	16	4	2

Table 16 shows that patients with severe Dengue and Dengue with warning signs had more than 4 days of fever in primary and secondary Dengue, indirectly indicating

viremia. Persistent viremia was associated with severity of diseases. Patients without warning signs had less than 4 days of fever in primary and secondary Dengue.

Table 17: Association between serological classification of Dengue and Sex

Serological test	**Male**	**Female**	**Pearson Chi-square Value**	**'P' Value**
Dengue Negative	11 (14.4%)	21 (27.6 %)	3.649	0.161 (NS)
Primary Dengue infection	35 (47.3%)	31 (40.8 %)		
Secondary Dengue infection	28 (38.1)	24 (31.6%)		

Table 17 shows that there was no significant difference in male to female ratio in primary, secondary and serological negative Dengue cases (P value: 0.161).

Table 18: Association between serological classification and liver enzyme level

Serological classification	**Liver enzyme not done**	**Liver enzyme normal**	**Liver enzyme elevated**	**Pearson Chi-square Value**	**'P' Value**
Negative	14 (82.4%)	11 (15.1%)	7 (11.7 %)	51.42	0.00 (S)
Primary Dengue	3 (17.6%)	41 (56.2%)	22 (36.7%)		
Secondary Dengue	0	21 (40.4%)	31 (51.6 %)		

Table 18 shows a strong association between elevated liver enzymes and serological classification, more in secondary Dengue 59.6 % (31 cases) when compared to primary Dengue 36.7 % (22 cases) (P value: 0.00).

Table 19: Association between platelet counts and WHO classification of Dengue

Serological Classification	Platelet Count < 150X10^3	Platelet Count 150-400 X 10^3	Platelet Count > 400 X10^3	Total	Pearson chi-square value	P value
Negative	**8 (25%)**	**23 (71.9 %**	**1 (3.1%)**	**32**	**44.100**	**0.00 (S)**
Primary Dengue	**46 (69.7%)**	**19 (28.8 %)**	**1 (1.5 %)**	**66**		
Secondary Dengue	**47 (90.4%)**	**4 (7.7 %)**	**1 (1.9 %)**	**52**		

Table 19 shows that there was a strong association between thrombocytopenia and clinical severity of the infection. (P value: 0.00). Thrombocytopenia was seen in 90.4 % (47 cases) of secondary Dengue, 69.7 % (46 cases) of primary Dengue and even seen in 25 % (8 cases) of sero-negative patients.

ISOLATION OF DENGUE RNA:

Dengue viral RNA was extracted by 1. QIA symphony DSP virus/pathogen midi kit,2. QIA symphony DSP virus/pathogen mini kit ,3. Roche kit and 4.QIAGEN kit, of which the midi kit was the best method which is shown in figure 14.

RNA PURITY AND CONCENTRATION CHECK:

Isolated RNA was subjected to spectrophotometry at wavelengths of 260 and 280($A_{260,}$ A_{280}). Twenty two samples with more than 50 NS1 antigen units were subjected

to RNA extraction by QIAsymphony midi kit which showed more than 40 microgram per milliliter of RNA, which was subjected to PCR.

ONE STEP MULTIPLEX-NESTED RT-PCR:

Out of 22 samples subjected to one step multiplex Nested RT- PCR, 15 samples showed Dengue amplicons with base pair 119(serotype2), base pair 290(serotype3) and base pair 119&290(serotype 2&3) as shown in figures 16,17,18 respectively.

Table 20: Distribution of Dengue serotypes in study group

Base pair size	Serotypes	Total positivity	Percentage (%)
119	DENV-2	4	18.18
290	DENV-3	5	22.73
119&290	DENV-2&3	6	27.27

Figure 13: Distribution of Dengue p

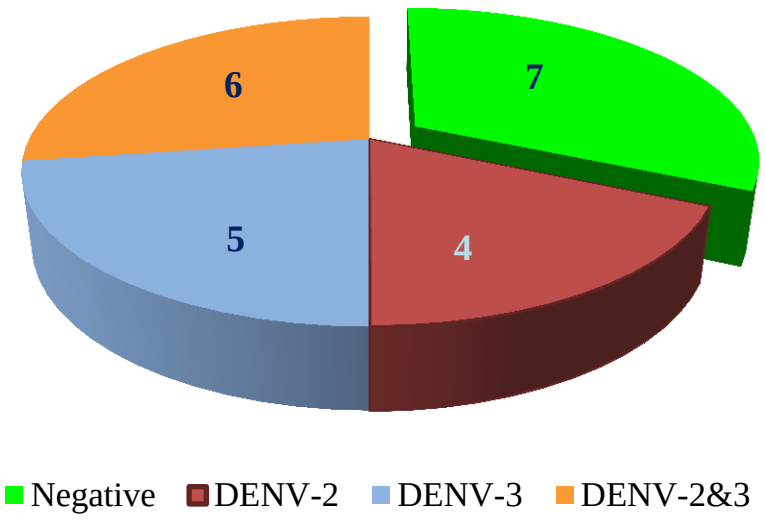
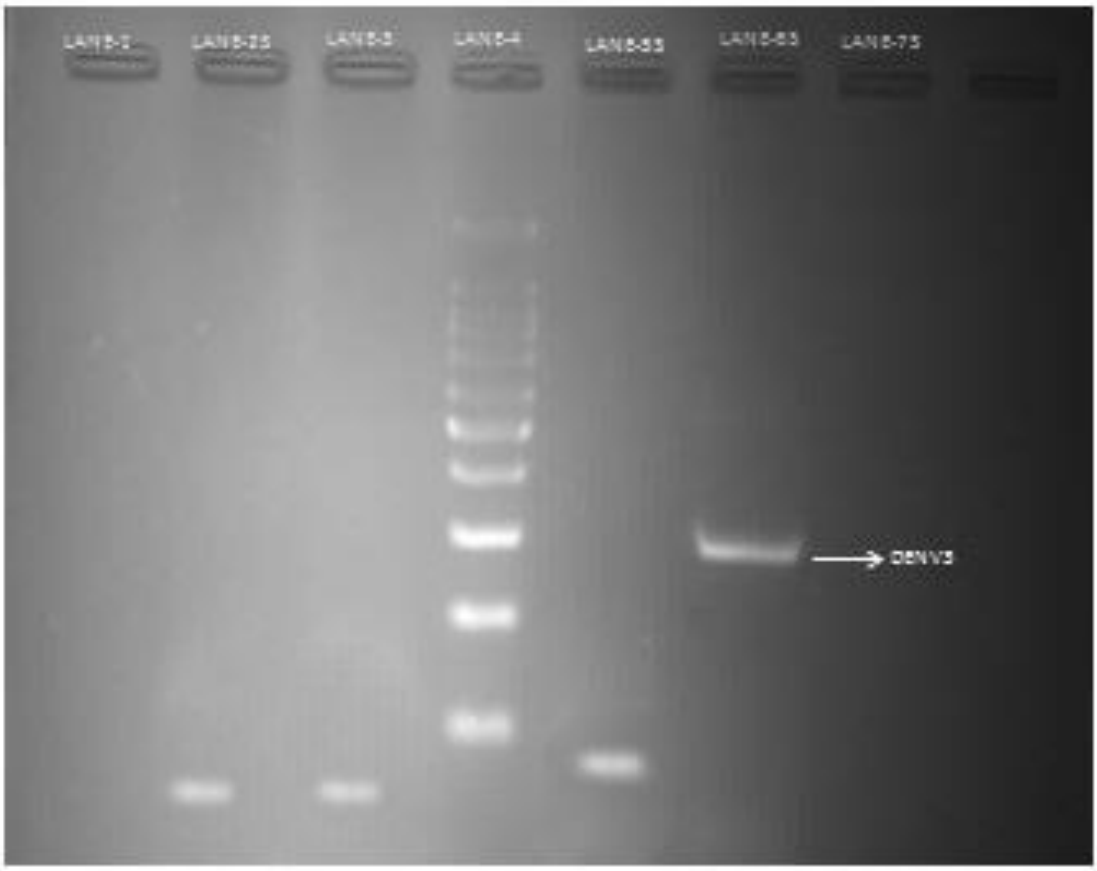


Table 20 shows that the most common serotype identified was DENV-2 and DENV-3 co-infection 27.27%(6) followed by DENV-3 22.73%(5) and DENV-2 18.18%(4).

Figure 14: Electrophoresis of Extracted RNA by various methods



Lane-2S => Manual extraction by QIAGEN RNA extraction kit

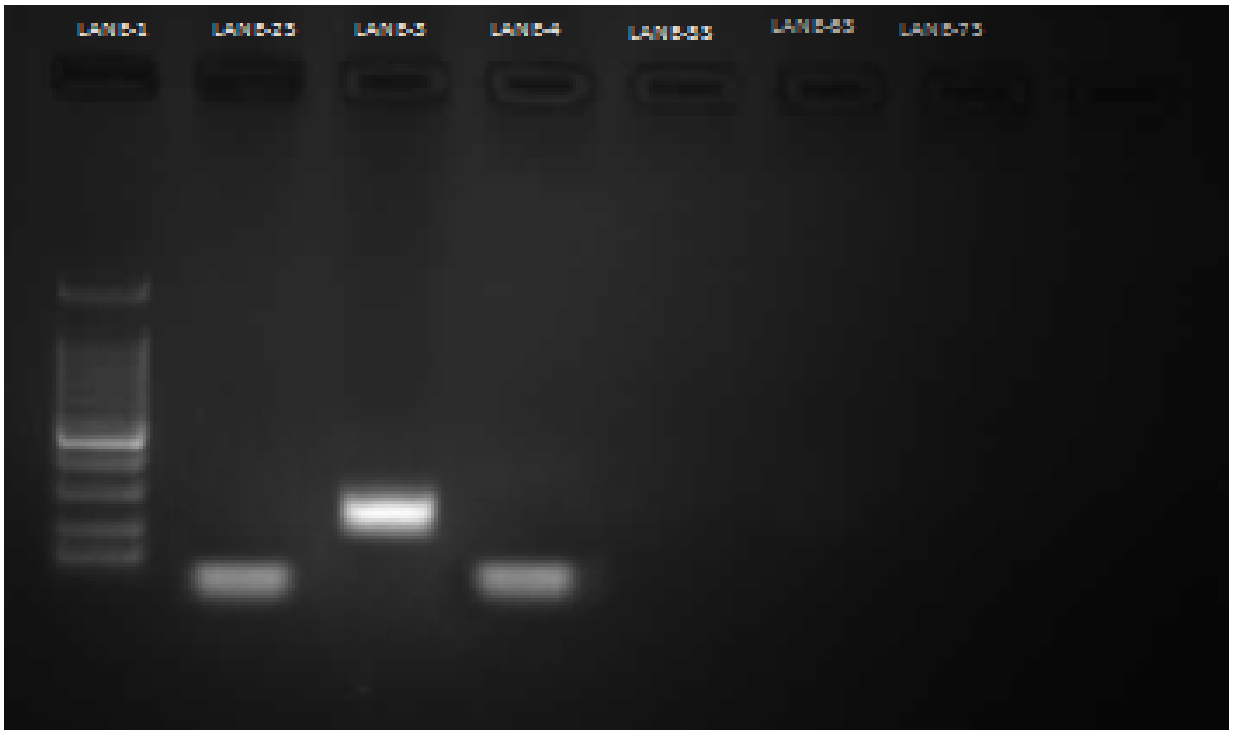
Lane-3S=> Manual extraction by QIAGEN RNA extraction kit

Lane-4=> 100bp molecular marker

Lane-5S=> QIA symphony DSP virus / Pathogen mini kit

Lane-6S => QIA symphony DSP virus / Pathogen midi kit

Figure 15: MOBS buffer Gel Electrophoresis using formaldehyde for Purification of extracted RNA identification

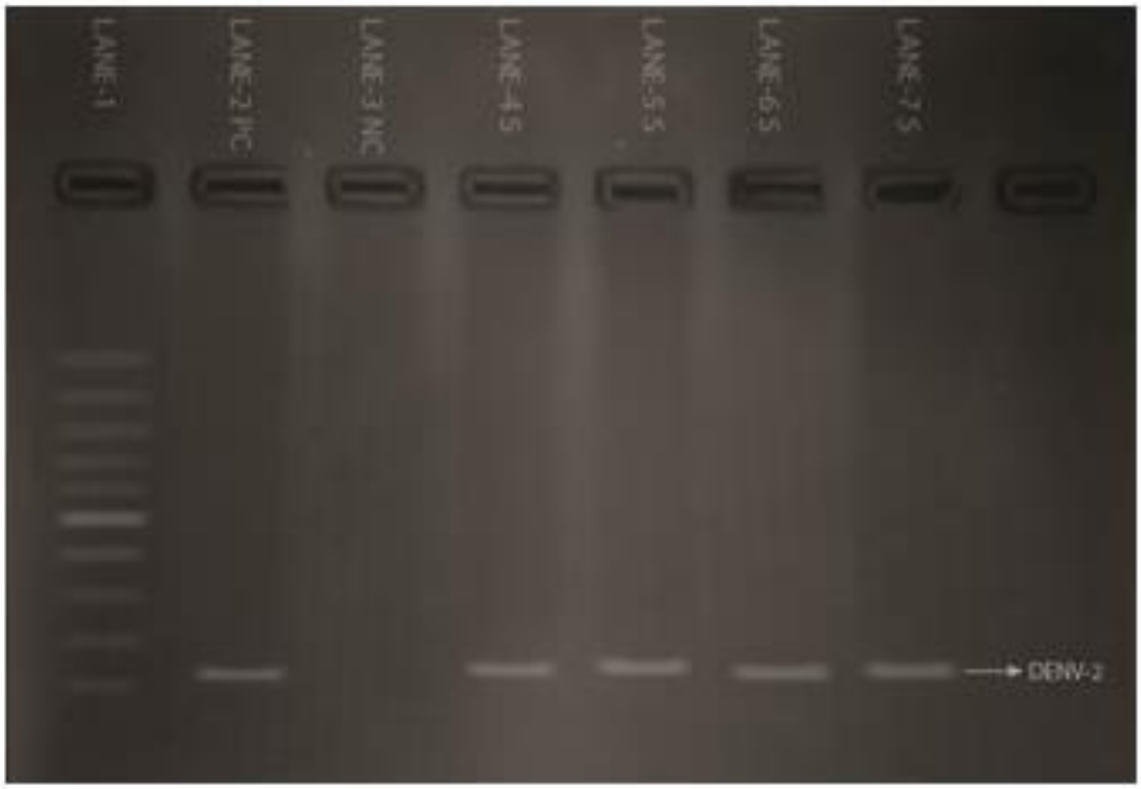


Lane-1 DNA Marker (100bp)

Lane-3 showed Dengue Viral RNA.

Lane – 1&3 showed mRNA

Figure 16: Result of Gel Electrophoresis of Dengue Serotype-2



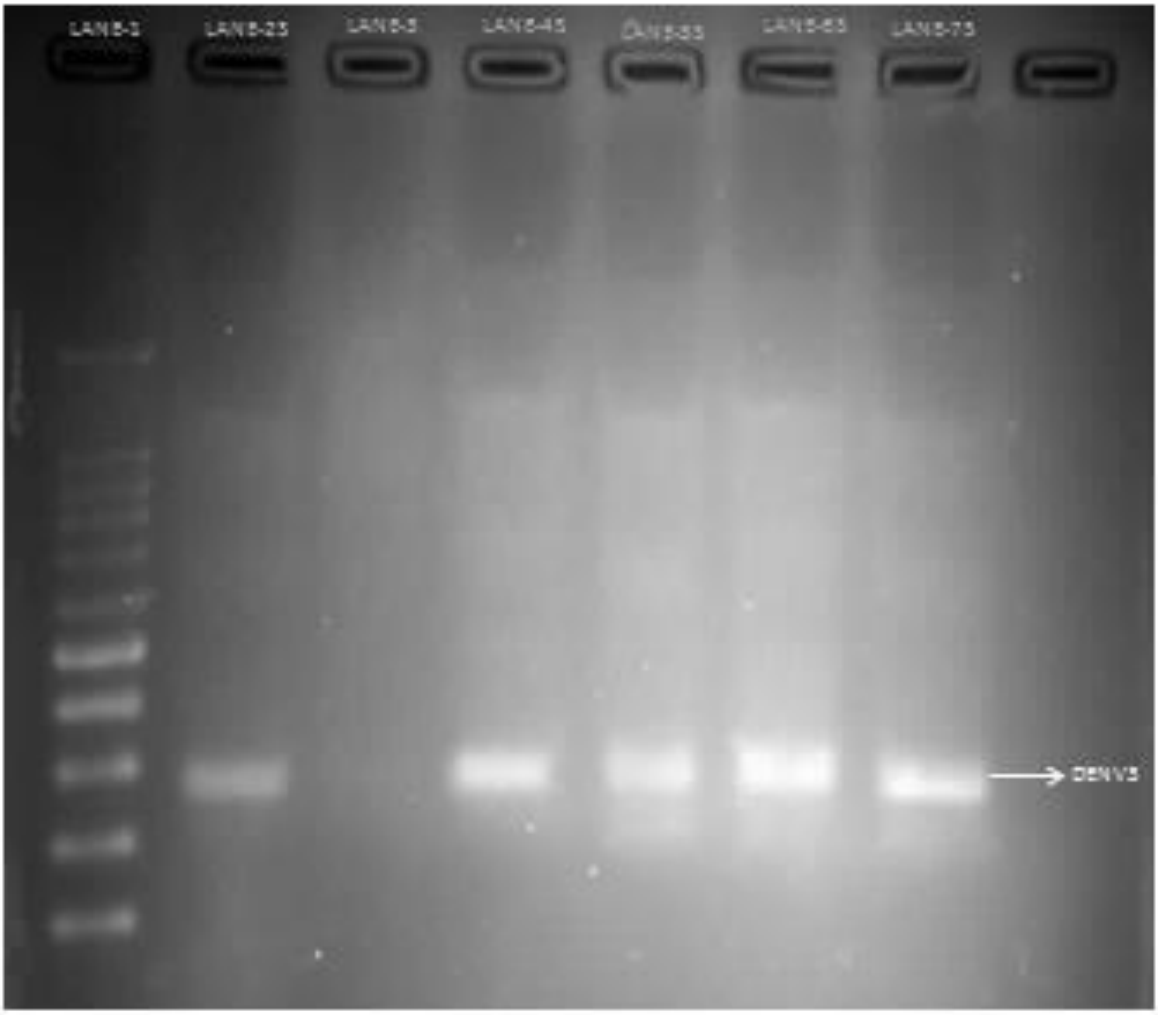
Lane-1 100 base pair Molecular marker

Lane-2 Positive Control (known DENV2 sample

Lane-3 Negative Control (known Dengue seronegative sample

Lane-4 45, 55, 65 & 75 = Dengue Seropositive samples, Positive for DENV2 (119bp)

Figure 17: Results of Gel Electrophoresis of Dengue Serotype-3



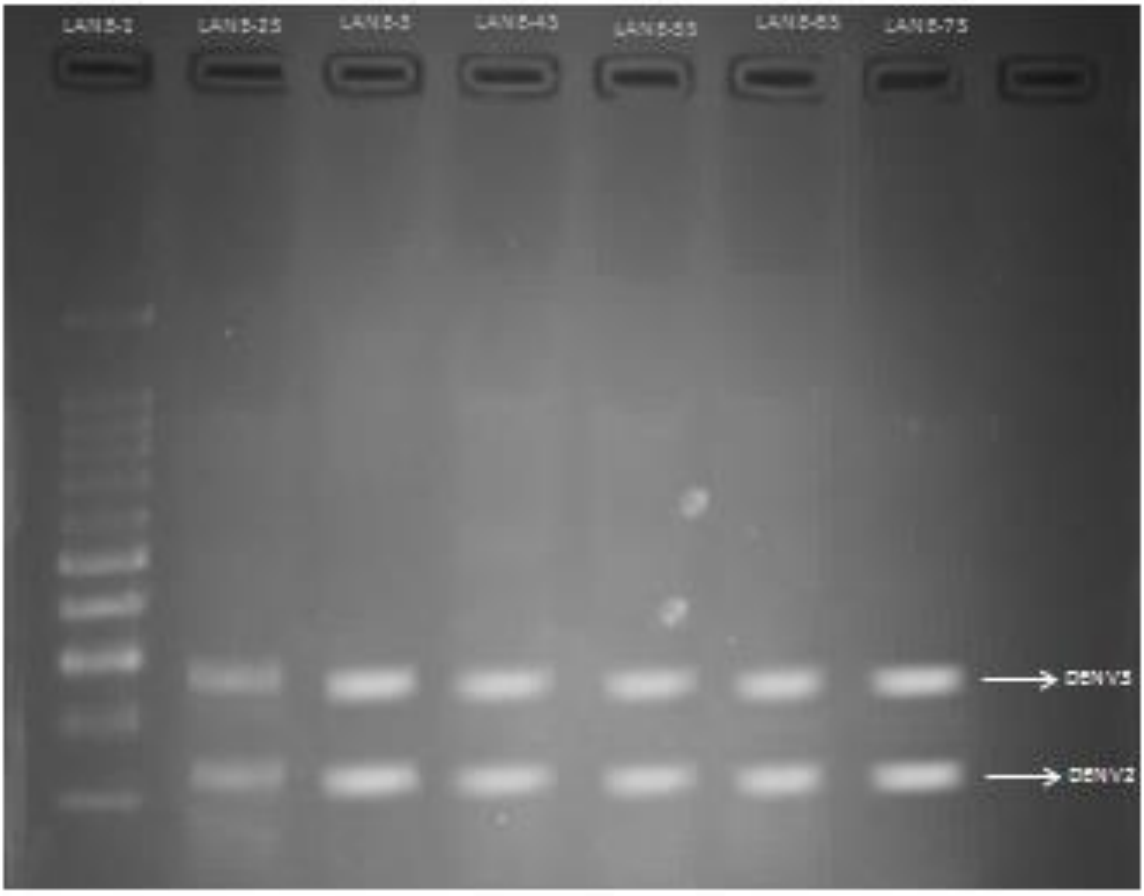
Lane-1 100 base pair Molecular marker

Lane-3 Negative Control (known Dengue seronegative sample

Lane-2S, 4S, 5S, 6S & 7S = Dengue Seropositive samples, Positive for DENV3 (290bp)

Lane-2 Positive Control (known DENV2 sample

Figure 18: Results of Gel Electrophoresis of Dengue Serotype-2&3



Lane-1 100 base pair Molecular marker

Lane-25, 35, 45, 55, 65 & 75 = Dengue Seropositive samples, Positive for DENV3 (290bp) and DENV-2 (119bp)

Figure 19: QIAGEN RNA Extraction (Automatic)

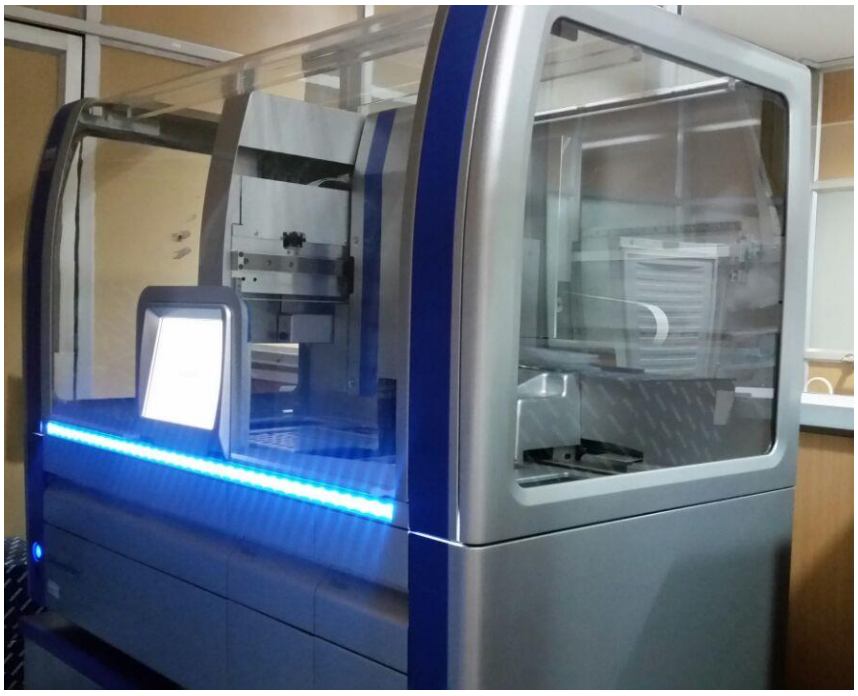
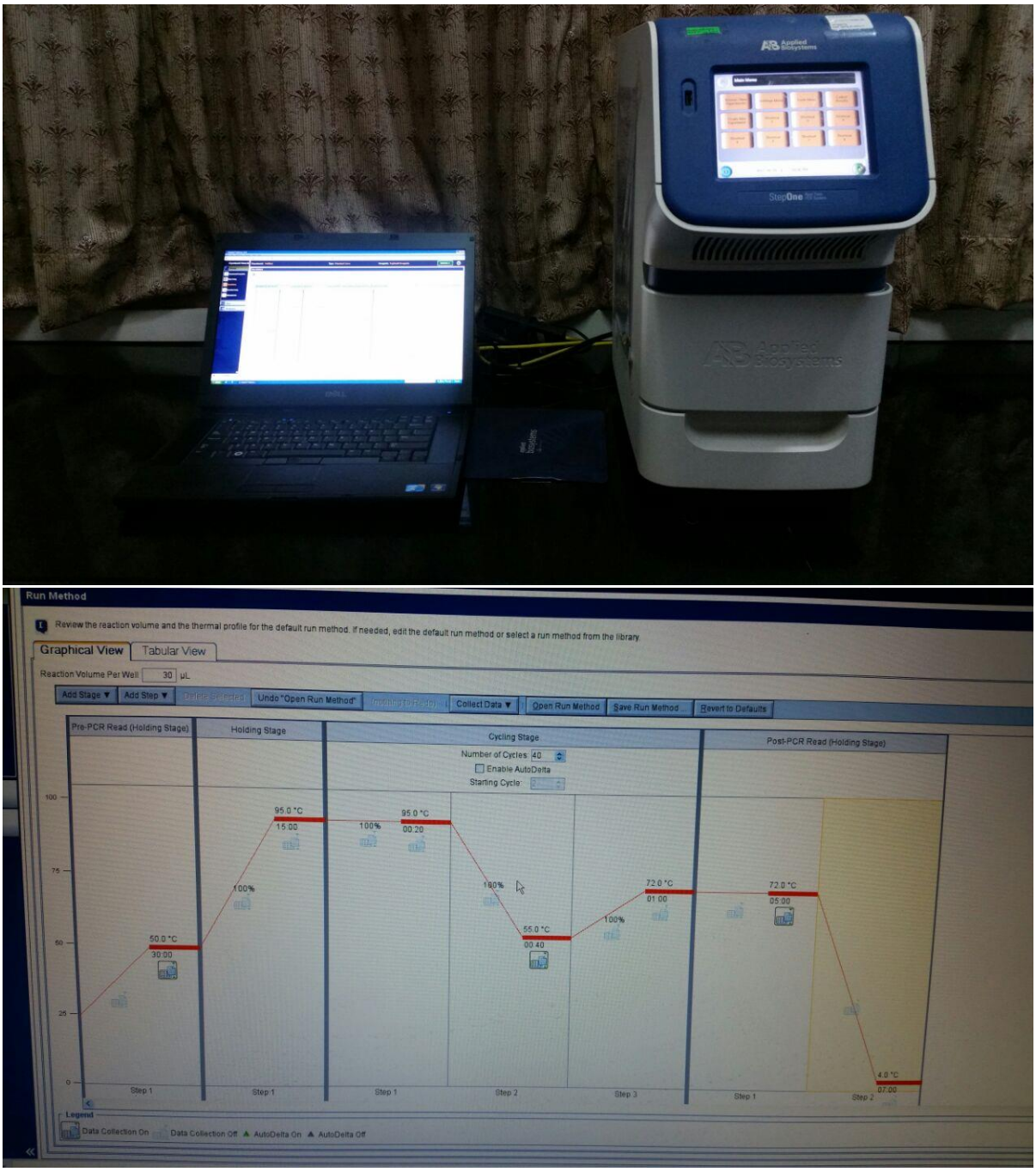


Figure 20: Spectrophotometer to deduct RNA Concentration

Figure 21: ABI Step-1 Real-time PCR Thermo Cycler



Thermocycler- PCR cycle

DISCUSSION

Dengue is one of the major mosquito borne viral disease with rapid rise in incidence in the last 50 years. Dengue infects an estimated 50 to 100 million people and responsible for increase in hospitalization and deaths. Since more than half of the infected individuals presents as asymptomatic or mild undifferentiated fever, the diagnosis is primarily based on the combination of clinical features, serology and molecular testing. According to National Vector Borne disease Control Programme-(Delhi) statistics as on 8th October 2017 the total Dengue positive cases all over India were 78691 out of which number death were 122and in Tamilnadu total cases were 11552 out of which number of death were 18.

Dengue infection may be primary or secondary. Primary infection can be subclinical or manifests as minor symptoms in most of the affected individuals. Secondary infection is more commonly associated with severe disease manifestations like Dengue haemorrhagic fever or Dengue shock syndrome. In primary Dengue infection the earliest antibody response is IgM appearing by third day, followed by IgG in later phase (6 to 15 days) of the disease persisting for more than six months following infection. In secondary Dengue infection, IgG antibodies will be detected even during the early acute phase, whereas IgM will be low or absent. Hence the presence of IgM antibodies in earlier phase of the disease indicates primary infection and presence of IgG indicates secondary infection.

Viral non-structural antigen (NS1) is abundant in the serum during earlier stages of infection; therefore NS1 antigen ELISA along with IgM capture ELISA will be more

effective during an endemic setting. In a study conducted by King institute, Chennai[82] during 2006 to 2008 showed 43% Dengue seropositivity with 16.47% being secondary infections. Another study conducted in Bangalore by Atul Garg et al[83] between 2006 to 2010 showed maximum seropositivity during 2010 (46.5%) and 92% were secondary infections. In our study the seropositivity over the years was 78.67% with 55.9% primary Dengue and44.1%secondary Dengue. But during the study period of one year, secondary Dengue found to be common than primary.

Generally there is no significant difference in male to female ratio of Dengue infection[43]. In a study conducted by Vemu Lakshmi et al[65], the male to female ratio was 2:1 during 2007. Another study by Tina Damodar et al[84] in Mangalore showed that male to female ratio was 2:1. In our study, male to female ratio was 1.2:1.As Dengue prevalence increases all part of our country over the years the ratio of affecting male and female are equal.

Commonly affected age group in our study was 11to 20 years (32%), followed by 21 to 30 years (18.7%) with mean age of 26 years.Arti Sharma et al from Madhya Pradesh showed 11 -20 was most common followed by21-30 years. Study by Meena Dias and Tina Damodar et al[84]. the most commonly affected age group was between 21 to 40 years (54%), followed by 10 to 20 years (19%). M. Neeraja et al[85] showed that the most commonly affected age group was between 20 to 39 years.

Numbers of Dengue cases are more during the monsoon and post monsoon season. Tamilnadu receives maximum rainfall from North East monsoon during October to

November .during the south west monsoon (SWN) from June to September Coimbatore district receives 32% of total annual rainfall of Tamilnadu.In our study, maximum number of cases were seen between the months of July (23.4%) and August (19.5%). In a laboratory based Dengue surveillance study conducted by John Victor et al[86] in 2007, the data on month wise incidence of Dengue in Tamilnadu for the past nine years revealed that the number of cases increased from June to December. PM Ukey et al[87]observed maximum Dengue cases in central part of India during September to November.

We observed IgM in 22.45%, IgM&IgG in 26.20% and IgG in 22.16%, indicating predominance of secondary Dengue infection than primary. Similar observations were made in another study from south India with 18% IgM positivity, 32% IgG positivity and 50% IgM and IgG positivity.

The Dengue virus NS1 antigen was found to be circulating in the sera of patients during the acute phase of illness from day 1 to day 9[43]. It has both group specific and type specific determinants. The NS1 antigen capture ELISA has been shown to be useful in determining Dengue infection in acute phase sera during both primary and secondary infections, although the sensitivity of detection is higher in primary infections. A possible explanation for reduced NS1 antigen sensitivity during secondary Dengue is due to the presence of anti-DENV antibody, plasma NS1 antigen sequestrated in immune complexes and the target epitopes are not accessible for its detection[43].In a study by Damodar T et al[84]. NS1 antigen was detected in 49% of patients with less than 5 days of fever. Another study by S Datta and Wattal[8] found that 71.42% NS1 antigen positivity

when samples were collected within 5 days of fever. In our study of selected 150 consecutive patients NS1 antigen positivity was 55.93 %(66). NS1 antigen is detected in 68.2% of patients with less than 5 days of fever and in 31.8% patients with more than 5 days of fever.

Normal platelet count is 150 to $400x10^{3.}$ In majority of Dengue patients thrombocytopenia is transient and asymptomatic. But in significant number of cases there are bleeding manifestations. Thrombocytopenia is due to immune mediated platelet destruction. When platelet counts are in the range of 20 to 40 thousands, petechiae/purpura is seen and when counts are less than 20 thousands, spontaneous bleeding is seen. Most commonly observed significant laboratory parameter in Dengue is thrombocytopenia which we observed in 69.7% of primary Dengue and 90.45% of secondary Dengue.

Normal PCV is in the range of 40-45 % for men and 35-40 % for women. Significantly elevated PCV were seen in severe Dengue, more in cases of secondary Dengue infection in our study (100%). Plasma leakage is evidenced by increase in hematocrit equal to or more than 20% above average for age, sex and population or signs of plasma leakage such as pleural effusion, ascites and hypoproteinemia.

Liver enzymes such as aspartate aminotransferase and alanine aminotransferase are commonly elevated in Dengue infections. Other enzymes like alkaline phosphatase and gamma glutamyl transpeptidase are also elevated in minority of patients[83].Significant

elevated liver enzymes were found in 36.7% of primary infection and in 51.6%secondary Dengue. Similar observation was made in studies by Damodar T et al[84].

Molecular techniques allows for multi-fold biological amplification of viral RNA and has been used for rapid diagnosis of viral diseases. The main advantage is faster screening, high sensitivity and specificity and can detect even small quantities of virus. This method is also used to detect the Dengue virus serotyping both in clinical samples and in Aedes mosquitoes. RT-PCR is the gold standard test one of the important steps in the molecular diagnosis of Dengue virus[80].

Extraction is an important prerequisite for a successful molecular detection of any viral pathogen.We used four methods for RNA extraction. They were 1). QIA symphony DSP virus/pathogen midi kit for RNA extraction, 2). QIA symphony DSP virus/pathogen mini kit for RNA extraction, 3). Roche kit and 4).QIAGEN kit.

Out of which QIA symphony DSP virus/pathogen midi kit yielded higher concentrations of RNA than the other methods. Further purification of RNA was achieved by spectrophotometry. All samples showed more than 40µg/ml of RNA. Extracted RNA was subjected to MOBS buffer Electrophoresis using formaldehyde gel to detect and differentiate Dengue RNA from non-specific microRNA normally present in serum.

Dengue viral RNA was amplified by various methods. They were Reverse transcriptase PCR(RT-PCR),Real time PCR(qRT-PCR), Transcription mediated amplification(TMA), Nucleic acid sequence based amplification(NASBA) and loop

mediated isothermal amplification(LAMP).In RT-PCR CprM(Core pre-Membrane) structural region was amplified for identification of Dengue virus. We used single step multiplex nested RT-PCR, using the primers designed by Lanciotti et al[66]. The primers used were as follows

D1-5'-TCAATATGCTGAAACGCGCGAGAAACCG-3'-511
D2-5'-TTGCACCAACAGTCAATGTCTTCAGGTTC-3'-511
TS1-5'-CGTCTCAGTGATCCGGGGG-3'-482 (D1 & TS1),
TS2-5'-CGCC ACAAGGGCCATGAACAG-3'-119 (D1 & TS2),
TS3-5'-TAACATCATCATGAGACAGAGC-3'-290(D1&TS3)
TS4-5'-CTCTGTTGTCTTAAACAAGAGA-3'-392 (D1 & TS4)

The advantages of this test is its better sensitivity and accurate detection of viral RNA for a specific serotype. The main disadvantage is its cost and requires expertise. We could not use real time PCR as it is very expensive.

There are five serotypes of Dengue virus, DENV 1 to 5, fifth being the latest addition. The fifth and the latest serotype have been declared in October 2013. This serotype was found in the sample of a 37 year old farmer from Sarawak state of Malaysia in 2007[79]. Most of the infections are due to serotypes 1 to 4 with increase in incidence of cross infection among the serotypes. Increase in cross infection leads to the emergence of recombinant strains and disease severity.

Among the serotypes of Dengue virus, Asian serotypes of DENV-2&3 are frequently associated with secondary Dengue infection as observed by Tina Damodar et

al[84]. Neeraj et al showed that the common serotype was DENV-3 followed by DENV-4 in southern part of India. In our study DENV-3 was the most common followed by DENV-2. However DENV-2 and DENV-3 coinfection was seen in 27.27% of cases followed by DENV-3 in 22.73% and DENV-2-18.88%.

Fifty percent of coinfection with DENV-2 and DENV-3 in our study were associated with increase in disease severity leading to Dengue haemorrhagic fever and Dengue shock syndrome[81]. 33.33%(2 cases) of coinfection had Dengue fever with warning signs and 16.66%(1 case) had Dengue fever without warnings. These serotypes were observed to circulate in this part of country in the current season of Dengue. However more molecular data were required to determine the true picture as this was only a small sample study.

The treatment of Dengue virus infection is essentially supportive and symptomatic. No specific treatment is available and relies on WHO guidelines. Patients with mild symptoms were managed by maintaining oral hydration and monitored for warning signs. Patients with warning signs were managed with intravenous fluids after obtaining the haematocrit values and hydration was titrated accordingly. Patients with shock and haemorrhage were managed with intravenous crystalloid solution and blood transfusion.

SUMMARY

- The total number of patients with suspected Dengue in our hospital from August 2016 to August 2017 was 6557, out of which 70.81%(4643) were seropositive.
- A total of 150 consecutive serum samples were selected from August 2016 to August 2017, during peak Dengue season (August 2017) based on WHO clinical classification.All samples were subjected to NS1 microlisa, IgM capture ELISA and IgG capture ELISA(Panbio).Out of 150 samples, seropositivity was 78.67 %(118) with 55.9%(66) primary Dengue and 44.1%(52) secondary Dengue infections.
- IgM positivity was 9.3%(11), NS1+IgM was 9.3%(11), NS1+IgM+IgG was also 9.3%(11), IgM+IgG was 33.99%(40) and only IgG was 0.85%(1).
- NS1 antigen positivity was found in 55.93% (66) out of 118 patients, most of them had less than five days of fever. Nine patients out of these 66 showed NS1 positivity (7.63%) on 9^{th} day of fever
- males were affected more than females(1.2:1).
- Commonly affected age group was11 to 20 years (32%).
- More number of cases were seen between the months of July (23.4%) and August (19.5%).
- Most common clinical symptom was high grade fever followed by joint pain.
- Most commonly observed significant laboratory parameters were a)Thrombocytopenia (69.7% in primary Dengue, 90.4% in secondary Dengue) b) Elevated liver enzymes (36.75% in primary Dengue, 59.6% in secondary Dengue).

- Molecular typing: Twenty two random samples had more than 50 NS1 antigen units, from which RNA was extracted by 1.QIA symphony DSP virus/pathogen midi kit, 2. QIA symphony DSP virus/pathogen mini kit, 3. Roche kit and 4.QIAGEN kit
- Out of which QIAsymphony midi kit yielded more concentration of RNA, which has been subjected to spectrophotometry to find out approximate concentration of RNA. All samples showed more than 40 µg/ml of RNA. Extracted RNA was subjected to MOBS buffer Electrophoresis using formaldehyde gel to detect and differentiate Dengue RNA from non-specific microRNA normally present in serum.
- After this extracted RNA were subjected to one step Multiplex Nested RT-PCR by using following primers

  D1-5'-TCAATATGCTGAAACGCGCGAGAAACCG-3'-511

  D2-5'-TTGCACCAACAGTCAATGTCTTCAGGTTC-3'-511

  TS1-5'-CGTCTCAGTGATCCGGGGG-3'-482 (D1 & TS1),

  TS2-5'-CGCC ACAAGGGCCATGAACAG-3'-119 (D1 & TS2),

  TS3-5'-TAACATCATCATGAGACAGAGC-3'-290 (D1 &TS4

  TS4-5'-CTCTGTTGTCTTAAACAAGAGA-3'-392 (D1 & TS4)

- Amplified products of RT-PCR were subjected to agarose gel electrophoresis for Dengue virus serotype identification. Fifteen out of 22 samples tested showed amplified product (68.18%).

- The most common serotype identified was DENV-3(11cases) followed by DENV-2(10cases) among which Co-infection with DENV 2&3was seen in 6 cases , followed by DENV-3 alone in 5 cases and DENV-2 alone in 4 cases.
- Fifty percent (3 cases) of DENV-2&3 coinfection was associated increase in disease severity leading to Dengue haemorrhagic fever and Dengue shock syndrome. 33.33%(2 cases) of coinfection had Dengue fever with warning signs and 16.66%(1 case) had Dengue fever without warning signs.
- Eighty three percent (5 cases) of DENV-2&3 coinfection was primary Dengue. 16.67%(1case) of coinfection was secondary Dengue.
- DENV-3 and DENV-2 infection was associated with Dengue fever without warning signs.

CONCLUSION

Dengue fever is a rapidly evolving mosquito borne viral disease in our community. Early diagnosis of the infection by the detection of NS1 antigen coupled with capture ELISA followed by RT-PCR for serotype identification leads to proper treatment and reduction in morbidity and mortality. There are more primary infections than secondary infections. Little cross immunity exists between the serotypes offering less protection during cross infection leading to severe manifestations of the disease. The prevalent serotype of Dengue in this area was DEN-2&3 coinfection. Also infection with multiple serotypes leads to the emergence of recombinant strains..

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APPENDIX

MATERIALS REQUIRED (REAGENTS USED):

TBE Buffer:

- Tris base(89.00)- 10.78g
- Boric acid(89.00)- 5.5g
- EDTA(0.5)- 3.72g
- pH- 8.3

10X buffer preparation:

Mix the above contents in 1000 ml of milli Q water.

1X buffer preparation:

Nine hundred ml of milli Q water is added to 100 ml of 10X buffer.

Ethidium bromide:

10 mg/ml in distilled water.

2% Agarose gel preparation:

Two gram agarose powder, 50X TAE 2 ml, milli Q water 98 ml, ethidium bromide 0.4µl.

RNA Gel Run:

Agarose 1.2g, miili Q water 90 ml, 10X MOBS buffer 10 ml, formaldehyde 1.8 ml, ethidium bromide 0.341µl

ANNEXURE

ABBREVIATIONS

DENV	Dengue virus
DHF	Dengue haemorrhagic fever
DSS	Dengue shock syndrome
NS	Non-structural protein
DC	Dendritic cells
IL	Interleukin
IFN	Interferon
TNF	Tissue necrosis factor
CTLA	Cytotoxic T-lymphocyte associated protein
DC-SIGN	Dendritic cell- Specific intercellular adhesion molecule-3-Grabbing Non-integrin
TGF	Transforming growth factor
MMP	Matrix metalloproteinases
ICAM	Intercellular adhesion molecule
VCAM	Vascular cell adhesion molecule
VEGF	Vascular endothelial growth factor
PCR	Polymerase chain reaction
HRP	Horseradish peroxidase

PSG Institute of Medical Sciences & Research
Institutional Human Ethics Committee

Recognized by The Strategic Initiative for Developing Capacity in Ethical Review (SIDCER)
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Phone : 91 422 - 2598822, 2570170, Fax : 91 422 - 2594400, Email : ihec@psgimsr.ac.in

To
Dr P Thenmozhi
Postgraduate
Department of Microbiology
Guide: Dr B Appalaraju
PSG IMS & R
Coimbatore

Ref: Project No.15/412

Date: December 30, 2015

Dear Dr Thenmozhi,

Institutional Human Ethics Committee, PSG IMS&R reviewed and discussed your application dated 21.12.2015 to conduct the research study entitled *"Molecular detection of dengue virus serotypes from clinical samples prevalent in and around Coimbatore"* during the IHEC meeting held on 24.12.2015.

The following documents were reviewed and approved:

1. Project Submission form
2. Study protocol (Version 1 dated 21.12.2015)
3. Confidentiality statement
4. Application for waiver of consent
5. Data collection tool (Version 1 dated 21.12.2015)
6. Current CVs of Principal investigator, Co-investigators
7. Budget

The following members of the Institutional Human Ethics Committee (IHEC) were present at the meeting held on 24.12.2015 at IHEC Secretariat, PSG IMS & R between 10.00 am and 11.00 am:

Sl. No.	Name of the Member of IHEC	Qualification	Area of Expertise	Gender	Affiliation to the Institution Yes/No	Present at the meeting Yes/No
1	Mr. R. Nandakumar	BA., BL	Legal Expert, Chairperson	Male	No	Yes
2	Dr. S. Bhuvaneshwari (Member-Secretary, IHEC)	MD	Clinical Pharmacology	Female	Yes	Yes
3	Dr. S. Shanthakumari	MD	Pathology, Ethicist	Female	Yes	Yes
4	Dr D Vijaya	M Sc., Ph D	Basic Medical Sciences (Biochemistry)	Female	Yes	Yes

The study is approved in its presented form. The decision was arrived at through consensus. Neither PI nor any of proposed study team members were present during the decision making of the IHEC. The IHEC functions in accordance with the ICH-GCP/ICMR/Schedule Y guidelines. The approval is valid until one year from the date of sanction. You may make a written request for renewal / extension of the validity, along with the submission of status report as decided by the IHEC.

PSG Institute of Medical Sciences & Research
Institutional Human Ethics Committee
Recognized by The Strategic Initiative for Developing Capacity in Ethical Review (SIDCER)
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Phone : 91 422 - 2598822, 2570170, Fax : 91 422 - 2594400, Email : ihec@psgimsr.ac.in

Following points must be noted:

1. IHEC should be informed of the date of initiation of the study
2. Status report of the study should be submitted to the IHEC every 12 months
3. PI and other investigators should co-operate fully with IHEC, who will monitor the trial from time to time
4. At the time of PI's retirement/intention to leave the institute, study responsibility should be transferred to a colleague after obtaining clearance from HOD, Status report, including accounts details should be submitted to IHEC and extramural sponsors
5. In case of any new information or any SAE, which could affect any study, must be informed to IHEC and sponsors. The PI should report SAEs occurred for IHEC approved studies within 7 days of the occurrence of the SAE. If the SAE is 'Death', the IHEC Secretariat will receive the SAE reporting form within 24 hours of the occurrence
6. In the event of any protocol amendments, IHEC must be informed and the amendments should be highlighted in clear terms as follows:
   a. The exact alteration/amendment should be specified and indicated where the amendment occurred in the original project. (Page no. Clause no. etc.)
   b. Alteration in the budgetary status should be clearly indicated and the revised budget form should be submitted
   c. If the amendments require a change in the consent form, the copy of revised Consent Form should be submitted to Ethics Committee for approval
   d. If the amendment demands a re-look at the toxicity or side effects to patients, the same should be documented
   e. If there are any amendments in the trial design, these must be incorporated in the protocol, and other study documents. These revised documents should be submitted for approval of the IHEC and only then can they be implemented
   f. Any deviation-Violation/waiver in the protocol must be informed to the IHEC within the stipulated period for review
7. Final report along with summary of findings and presentations/publications if any on closure of the study should be submitted to IHEC

Kindly note this approval is subject to ratification in the forthcoming full board review meeting of the IHEC.

Thanking You,

Yours Sincerely,

SECRETARY
PSG IMS&R
COIMBATORE-641004.
INSTITUTIONAL HUMAN ETHICS COMMITTEE

Dr S Shanthakumari
Member
Institutional Human Ethics Committee

PSG Institute of Medical Sciences & Research
Institutional Human Ethics Committee

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December 30, 2015

To
Dr P Thenmozhi
Postgraduate
Department of Microbiology
PSG IMS & R
Coimbatore

The Institutional Human Ethics Committee, PSG IMS & R, Coimbatore -4, has reviewed your proposal on December 24, 2015 in its expedited review meeting held at IHEC Secretariat, PSG IMS&R, between 10.00 am and 11.00 am, and discussed your request to conduct the study titled:

"Molecular detection of dengue virus serotypes from clinical samples prevalent in and around Coimbatore"

The following are the suggestions / recommendations made by the reviewers:

- As blood collection is being done, consenting by the patient may be included
- Requested to mention age group of the study participants in the inclusion / exclusion criteria
- Requested to submit permission letter from Heads of Medicine and Paediatrics Departments

Decision: Approval subject to the verification of the above mentioned documents / modifications by IHEC.

Yours truly,

SECRETARY
PSG IMS&R
COIMBATORE-641004.
INSTITUTIONAL HUMAN ETHICS COMMITTEE

Dr Sudha Ramalingam
Alternate Member - Secretary
Institutional Human Ethics Committee

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